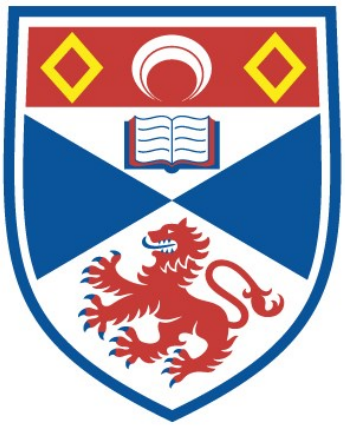
DIFFERENTIATION OF BONE CELLS IN VITRO

Cathy Wigzell

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1990

DIFFERENTIATION OF BONE CELLS *IN VITRO*

A Thesis

submitted to the University of St Andrews for the

Degree of Doctor of Philosophy

by

Cathy Wigzell

Department of Biology and Preclinical Medicine

University of St Andrews

August 1989

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

LIST OF CONTENTS

	page
Abstract	(i)
Declaration	(ii)
Copyright	(iii)
Acknowledgements	(iv)
Abbreviations	(v)
Figures	(vii)
Tables	(viii)
Summary	(x)
Introduction	1
Materials and Methods	39
Results	65
Discussion	106
References	126

ABSTRACT

Osteoblastic differentiation was studied *in vitro* using primary cultures of bone cells derived from neonatal mouse calvaria.

Using alkaline phosphatase as a marker, maintenance of the osteoblastic phenotype was found to be dependent upon the presence of ascorbic acid. No toxic effect due to ascorbic acid was seen. Insulin and dexamethasone were found to stimulate alkaline phosphatase expression, the former only in the absence of ascorbic acid. Two growth factors, epidermal growth factor and platelet-derived growth factor, were found to inhibit alkaline phosphatase expression in the presence of ascorbic acid.

Osteogenesis was most pronounced in cultures supplemented with ascorbic acid. The osteoblasts formed multilayers of cells and secreted an organic extracellular matrix composed mainly of type I collagen. Matrix vesicles were found among the collagen fibres. In the presence of ß-glycerophosphate, calcium phosphate crystals were deposited in discrete patches forming a mineralisation front which progressively engulfed osteoblasts. The type of matrix formed and the pattern of mineralisation resembled those of lamellar bone.

Insulin at 5000ng/ml stimulated matrix calcification in the absence of ascorbic acid. Dexamethasone, EGF and PDGF inhibited calcification. The extent of calcification was dependent upon the concentration of ß-glycerophosphate in the culture medium.

Conditioned medium from osteogenic cultures contained a GM-CSF which was secreted constitutively by the osteoblasts. Preliminary experiments with a mesenchymal stem cell line, Balb/c 3T3, showed the existence of a factor(s) with mitogenic activity in bone cell conditioned medium. No inducer of osteogenic differentiation was found.

DECLARATION

I hereby declare that the research reported in this thesis in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy is my own composition and is the result of work done by me during the period of matriculation for the above degree. No part of this work has been submitted previously for a higher degree.

The research was conducted in the Department of Biology and Preclinical Medicine, United College of St Salvator and St Leonard, University of St Andrews, under the supervision of Dr. C. W. Evans.

Signed Date 17.8.89

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No. 12 in September 1985, and as a candidate for the Degree of PhD in September 1985.

Signed Date 17.8.89

I hereby certify that Cathy Wigzell has spent nine terms engaged in research under my direction, and that she has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1 1967), and that she is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

Signed Date 17/8/89

COPYRIGHT

ACKNOWLEDGEMENTS

Naturally, I wish to thank my supervisors; Dr Clive Evans and Dr Jim Aiton. I would also like to express my appreciation and gratitude to the technical staff of the Department of Biology and Preclinical Medicine, especially Jean Anderson, Stella Campbell, David Ogden and Carol Voy.

ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
aFGF	Acidic Fibroblast Growth Factor
ALP	Alkaline Phosphatase
BDGF	Bone-derived Growth Factor
bFGF	Basic Fibroblast Growth Factor
BGP	Bone Gla Protein
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CFC	Colony Forming Cell
CFU-O	Colony Forming Cell - Osteogenic
CM	Conditioned Medium
CMF-PBS	Calcium/Magnesium Free Phosphate Buffered Saline
CSF	Colony Stimulating Factor
CT	Calcitonin
DNA	Deoxyribonucleic Acid
DOPC	Determined Osteogenic Precursor Cell
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EGF	Epidermal Growth Factor
FCS	Fetal Calf Serum
IBMX	3-Isobutyl-1-Methyl-Xanthine
IGF	Insulin-like Growth Factor
IOPC	Inducible Osteogenic Precursor Cell
MEM	Minimum Essential Medium
MGP	Matrix Gla Protein
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate Buffered Saline

PDGF	Platelet-derived Growth Factor
PTH	Parathyroid Hormone
SGF	Skeletal Growth Factor
TGF	Transforming Growth Factor
TRIS	Tris(hydroxymethyl) Methylamine

LIST OF FIGURES

Figure 1.	Lineage diagram showing the progression by proliferation and differentiation from a few, undifferentiated cells to the much larger population of terminally differentiated end cells.	4
Figure 2.	Lineage diagram showing the inter-relationships between the various members of the stromal compartment associated with marrow.	5
Figure 3.	Diagram describing the anatomy of the calvarial plates.	8
Figure 4.	Graph to show the effect of ascorbic acid upon alkaline phosphatase content.	72
Figure 5.	Graph to show the effect of ß–glycerophosphate upon alkaline phosphatase content.	73
Figure 6.	Graph to show the relationship between *p*-nitrophenol against alkaline phosphatase.	76
Figure 7.	Graph to show the change in alkaline phosphatase activity with time in culture.	79
Figure 8.	Graph to show the effect of EGF upon alkaline phosphatase activity.	83
Figure 9.	Speckle picture of the localisation of calcium in an osteogenic culture.	86
Figure 10.	X-ray emission analysis of a mineralised nodule.	87
Figure 11.	TEM from a 7 day old osteogenic culture.	88
Figure 12.	TEM from a 10 day old osteogenic culture.	88
Figure 13.	TEM of matrix vesicles amongst collagen fibrils in the extracellular space.	89
Figure 14.	TEM of banded collagen and nascent mineral spicules.	90
Figure 15.	TEM of an osteoblast in close proximity to mineral.	91
Figure 16.	TEM of an osteocyte in the centre of an osteogenic nodule.	92
Figure 17.	TEM of a rounded cell on the surface of a nodule.	93
Figure 18.	Phase contrast light micrograph of polygonal osteoblasts *in vitro*.	94
Figure 19a.	Light micrograph of an unmineralised osteogenic culture.	95
Figure 19b.	Light micrograph of a mineralised osteogenic culture.	95
Figure 20.	Graph to show the effect of insulin upon the extent of mineralisation.	97
Figure 21.	Graph to show the effect of epidermal growth factor upon the extent of calcification.	99
Figure 22.	Graph to show the effect of culture age upon the ability of the conditioned medium produced to stimulate colony formation.	103

LIST OF TABLES

Table 1.	Summary of the main molecules associated with the bone matrix.	18
Table 2.	Summary of the characteristics of the main growth factors found within the bone matrix.	25
Table 3.	Viability and number of cells produced during method II for cell preparation.	66
Table 4.	Stimulation of cAMP production in bone cells by calcitonin and parathyroid hormone.	67
Table 5.	Percentage primary cells positive for alkaline phosphatase extracte following method I.	68
Table 6a.	Table showing the alkaline phosphatase content and cell numbers of cultures of cells of the early population prepared following method II.	69
Table 6b.	Table showing the alkaline phosphatase content and cell numbers of cultures of cells of the late population prepared following method II.	70
Table 7.	Comparison using two way analysis of variance of supplemented cultures, in terms of alkaline phosphatase.	71
Table 8.	Comparison using two way analysis of variance of supplemented cultures, in terms of final cell number.	74
Table 9.	Alkaline phosphatase content and cell number following 14 days in culture with various concentrations of ascorbic acid.	75
Table 10a.	Effect of varying concentrations of ascorbic acid upon cell numbers and alkaline phosphatase activity following 7 days in culture.	77
Table 10b.	Effect of varying concentrations of ascorbic acid upon cell numbers and alkaline phosphatase activity following 14 days in culture.	78
Table 11.	Effect of organic phosphate upon cell numbers and alkaline phosphatase activity.	78
Table 12.	Effect of insulin upon cell numbers and alkaline phosphatase activity.	80
Table 13.	Effect of dexamethasone upon cell numbers and alkaline phosphatase activity.	81
Table 14.	Effect of EGF upon cell numbers and alkaline phosphatase activity.	82
Table 15.	Effect of PDGF upon cell numbers and alkaline phosphatase activity.	82
Table 16.	Data showing the effect of dexamethasone upon the extent of calcification.	96
Table 17.	Effect of organic phosphate upon cell numbers and calcium content.	100

Table 18.	Effect of seeding density on calcium deposition.	101
Table 19.	Proportion of GM-CFC colony types following stimulation with bone cell conditioned medium	102
Table 20.	Effect of bone cell conditioned medium upon Balb/c 3T3 cells.	105

SUMMARY

1. The differentiation of mouse osteoblasts was studied *in vitro*. Bone formation and the expression of alkaline phosphatase were used as markers of the differentiated phenotype. The effects of osteoblastic products upon haemopoietic and mesenchymal cells were also studied.

2. Primary cultures of osteoblasts were established following enzymatic digestion of neonatal mouse calvaria with dispase and collagenase. Three similar methods are compared.

3. Osteoblasts were found to be most abundant in neonatal mouse calvaria, using the expression of alkaline phosphatase as a marker. The periostea and sutures contained few osteoblasts. An initial method of sequential digestion of the calvaria revealed an increased release of osteoblasts from the bones with increased digestion time.

4. Post-confluent cultures (7 days old) of bone cells produced a strong response to parathyroid hormone in terms of cAMP production (140.5 ± 33.2 pmoles/10^6 cells). The response to calcitonin was negligible (3.1 ± 1.2 pmoles/10^6 cells).

5. Ascorbic acid is essential for the maintenance of alkaline phosphatase expression, up to 14 days in culture. In the absence of ascorbic acid there was a general decline in alkaline phosphatase expression with time in culture. There was no observable toxic action resulting from the addition of ascorbic acid. This was possibly due to the creation of hypoxic conditions as the cultures formed multilayers of cells.

6. Following the formation of multilayers, the cultures of osteoblasts developed osteogenic nodules. In the presence of ß-glycerophosphate the nodules mineralised. Transmission EM showed the extracellular matrix to be composed mainly of type I collagen. Matrix

vesicles were observed. Initial mineral deposition occurred in discrete patches in close association with collagen fibres. In the centre of the nodule, there were cells (osteocytes) embedded within the mineralised matrix and separated from this by a thin layer of osteoid.

7. In the absence of ascorbic acid, ß-glycerophosphate caused a significant increase in alkaline phosphatase expression. However, in the presence of ascorbic acid, ß-glycerophosphate caused a significant (but transient) decrease. ß-glycerophosphate was found to be necessary for the mineralisation of osteogenic nodules. The deposition of calcium increased in a dose-dependent manner with increasing concentrations of ß-glycerophosphate.

8. Only in the absence of ascorbic acid, did insulin cause a significant increase in alkaline phosphatase expression and matrix calcification with a concomitant decrease in cell proliferation. No changes in these parameters were seen in the presence of ascorbic acid. However, dexamethasone in the presence of ascorbic acid caused a transient increase in alkaline phosphatase expression over the 14 day culture period but was without effect on cell numbers. By day 14 the stimulation of alkaline phosphatase was no longer apparent and cell proliferation and the extent of calcification were both inhibited. In the absence of ascorbic acid, dexamethasone stimulated alkaline phosphatase expression at day 14.

9. In this system, EGF and PDGF were both found to lack mitogenic activity and to inhibit calcification. In the presence of ascorbic acid, both growth factors inhibited alkaline phosphatase expression. PDGF slightly stimulated alkaline phosphatase in the absence of ascorbic acid.

10. Media conditioned by osteogenic cultures were found to contain a factor(s) capable of stimulating the formation of colonies by GM-CFC in agar cultures. The activity was secreted constitutively and was probably GM-CSF.

11. Experiments with the mesenchymal stem cell line, Balb/c 3T3 clone A31, failed to produce any evidence for osteogenic potential. Culture of the cell line with media conditioned by osteogenic primary bone cells elicited a proliferative response, as opposed to one inducing differentiation.

INTRODUCTION

LIST OF CONTENTS

A. INTRODUCTION 2

B. STRUCTURE OF BONE
1. Cells of bone 2
i) Osteoblasts 2
a) *In vivo* 2
b) *In vitro* 7
ii) Osteoclasts 11
a) *In vivo* 11
b) *In vitro* 15
2. The extracellular matrix 18
i) The organic extracellular matrix 19
a) Collagen 19
b) Glycoproteins 20
c) Gla-containing proteins 21
d) Proteoglycans 22
e) Plasma proteins 23
f) Growth factors 23
ii) The inorganic extracellular matrix 26
a) The mineral phase 26
b) Mineralisation mechanisms 27

C. BONE FORMATION
1. Developmental bone formation 28
i) Endochondral osteogenesis 28
ii) Intramembranous osteogenesis 29
2. Bone repair 30
3. Experimental bone formation 30
i) *In vivo* bone formation 30
ii) *In vitro* bone formation 32
a) Organ culture 32
b) Cell culture 33
c) Marrow culture 35

A. INTRODUCTION

The differentiation of bone cells *in vitro* is characterised ultimately by the ability to form bone in a controlled manner. This study was undertaken to establish osteogenic cultures *in vitro* and to determine the influence of various factors upon bone differentiation, namely: ascorbic acid, organic phosphate, insulin, dexamethasone (a glucocorticoid) and growth factors. Since *in vivo* bone does not exist as an isolated tissue it was also of interest to study the interplay between bone cells and haemopoietic and pluripotent mesenchymal cells.

B. STRUCTURE OF BONE

The two main components of bone are the cellular compartment and the extracellular matrix. Bone is continuously being turned over; matrix is first resorbed by osteoclasts and then replaced by osteoblasts. This dynamic nature is coupled with a highly organised architecture indicating the existence of a complex regulatory mechanism. Bone tissue is responsive to systemic and local factors as well as mechanical stress and as such presents a very complicated model to study.

1. Cells of bone

There are two main cell types associated with bone, osteoblasts and osteoclasts. The sequential activity of these cells results in the steady and orchestrated turnover of the matrix while the terminal differentiation of osteoblasts (to osteocytes) provides an intricate cellular network within the bone matrix.

i) Osteoblasts

a) *In vivo*

The osteoblast is a polarised, cuboidal cell positioned on the matrix surface and is actively involved in the synthesis of matrix proteins and their subsequent uni-directional secretion onto the matrix surface (Rodan and Rodan, 1984). This cellular activity is reflected in the type of organelles found in the cytoplasm; abundant rough endoplasmic

reticulum for protein synthesis and a Golgi complex for protein packaging and secretion. Osteoblastic protein products include type I collagen, alkaline phosphatase, osteocalcin and osteonectin (Martin *et al.*, 1988).

Osteoblasts are found towards the end of a lineage derived from a stem cell which is part of the stromal compartment of bone marrow (Friedenstein *et al.*, 1987). Cells derived from colonies of bone marrow fibroblasts *in vitro* are capable of forming bone and cartilage following implantation *in vivo* in diffusion chambers, however, this has proved unrepeatable with similar cultures of human bone and marrow cells (Ashton *et al.*, 1985). Uncloned spleen stromal fibroblasts transplanted in diffusion chambers following culture *in vitro* will not normally form bone, the tissue formed being alkaline phosphatase negative and consisting of fibroblasts surrounded by collagen fibres (Friedenstein *et al.*, 1970). Stromal cells with spontaneous osteogenic ability are therefore to be found in marrow stroma, and not in stroma of non-marrow organs.

Having stated that non-marrow stromal cells are incapable of spontaneous osteogenesis, it should be noted that these cells can be **induced** to differentiate along the osteogenic pathway (Friedenstein *et al.*, 1970). Potent inductive stimuli have been found to be,

i) transitional epithelium, and

ii) decalcified bone matrix.

Osteogenic differentiation is maintained only in the continuing presence of the inducer and the stromal cells involved are referred to as Inducible Osteogenic Precursor Cells (IOPCs). IOPCs may also be found in marrow (Owen, 1980; Harada *et al.*, 1988). Marrow stromal cells following osteogenic differentiation need no such inducer and are consequently known as Determined Osteogenic Precursor Cells (DOPCs). Stem cells are undifferentiated cells of high mitotic potential that function as the source of lineages of differentiated cells resulting in the end cells, highly specialised terminally differentiated cells usually incapable of further division (Figure 1). DOPCs have the characteristics of stem cells; self-replication and the production of differentiated cells (Vaughan, 1981).

Stem cell -----> committed progenitors -----> differentiated end cells

Figure 1. Lineage diagram, showing the progression by proliferation and differentiation from a few, undifferentiated stem cells to the much larger population of terminally differentiated end cells.

Transplantation of marrow fragments results in osteogenesis at the new site followed by infiltration by haemopoietic cells of host origin (Friedenstein, 1976). The type of haemopoietic tissue formed is dependent upon the source of the transplant, ie red or yellow marrow (Patt *et al.*, 1982). Osteogenesis occurs in a similar manner, regardless of the marrow origin so the haemopoietic regulation is transferred by a set of marrow stromal cells. Retransplantation of the marrow component of the graft results in the formation of new osteogenic tissue. However, the number of possible retransplantations is very limited. There is therefore some particular element in bone marrow stroma that is capable of osteogenesis which is not found in the stroma of other organs. This indicates a finite ability of the stromal cells to self-maintain in an obvious absence of replenishment by host haemopoietic cells. Marrow transplanted from male to female mice survives and forms a new organ composed ostensibly of donor stroma and host marrow. Retransplantation of this organ to a secondary female host pre-immunised against male antigens results in resorption of the graft. If the transplant in the primary host had been repopulated with any host (female) stroma cells then the organ would survive transplantation to the secondary host (female). However, this does not happen and consequently all the stroma cells are male and therefore rejected by the pre-immunised secondary host (Friedenstein and Kuralesova, 1971). Therefore, despite the close structural and functional interplay between the osteogenic and haemopoietic compartments *in vivo*, the two lineages appear to self-maintain independently in the adult animal.

In correlation with the lineages of the haemopoietic system it has been suggested that the osteogenic system is composed of equivalent populations of stem cells, committed

progenitors and differentiating end cells (Owen, 1985). It is proposed that there is a pluripotent stromal stem cell capable of giving rise to committed progenitors for reticular, fibroblastic, adipocytic and osteogenic lineages (Owen and Ashton, 1986). Similar specialised stromal stem cells may exist in other systems (Figure 2). Following proliferation, the osteoprogenitor gives rise to the preosteoblast, an alkaline phosphatase positive, fibroblastic cell still capable of mitosis (Owen, 1980). The preosteoblast is the immediate precursor to the osteoblast, which has very little, if any, mitotic potential.

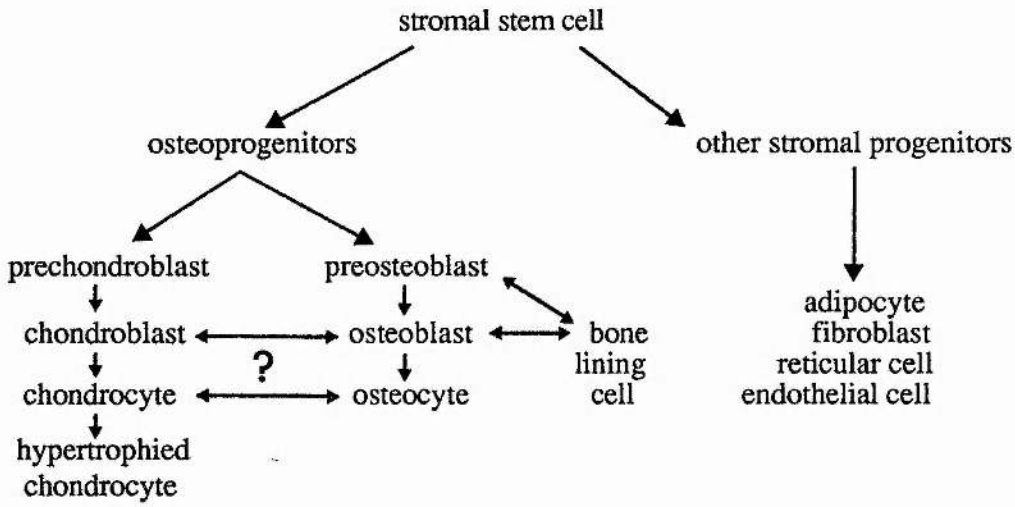


Figure 2. Lineage diagram showing the inter-relationships of the various members of the stromal compartment associated with marrow (after Marks and Popoff, 1988; Martin *et al.*, 1988).

A cell type possibly within the osteogenic lineage is the bone lining cell. This is a flattened cell directly apposed to the inactive bone surfaces which form a relatively large proportion of the total bone surface area (Miller and Jee, 1987). The cytoplasm is very thinly spread over the bone surface and contains a nucleus that is also very thin and is surrounded by those few organelles that are present; endoplasmic reticulum, free ribosomes and mitochondria (Miller *et al.*, 1980). Several functions have been attributed to bone

lining cells. They form a cellular membrane physically separating the bone fluids from the interstitial fluids and could therefore contribute to the regulation of mineral homeostasis by maintaining and regulating ion gradients (Bushinsky *et al.*, 1989). The bone lining cells form gap junctions between each other and also with osteocytes, raising the possibility of the existence of a 'functional syncytium' composed of bone lining cells and osteocytes; this may support the metabolism of osteocytes within the matrix. The bone lining cell may also be involved in the initiation of remodeling by;

i) attracting osteoclast precursors,
ii) retracting and therefore exposing the bone surface, and
iii) removing osteoid, ie the covering layer of unmineralised bone matrix.

It is also believed that the bone lining cell may represent an inactive osteoblast (Peck and Woods, 1988).

Osteocytes are the end cells of the osteogenic lineage. They lie within lacunae and are completely surrounded by the extracellular matrix. Both *in vivo* and *in vitro* osteocytes assume a stellate morphology by extending cytoplasmic processes. *In vivo* these protrude into canaliculi (Nijweide and Mulder, 1986). The osteocyte may contribute to mineral homeostasis by selective action (resorption and deposition) of the perilacunar bone (Chambers, 1980; Martin *et al.*, 1988). Due to the proximity of the osteocyte network with the bone mass, these cells may act as go-betweens, translating changes in bone stress to messages for osteoblast or osteoclast activation. Indeed the osteocyte may encourage osteoclastic resorption by secreting enzymes thus causing osteocytic osteolysis. This is seen during bone growth and also during pathological states, eg severe calcium deficiency (Chambers, 1980). In actively growing rabbit bone there is a fixed lifetime for the osteoblast while it is apposed to the bone surface and secreting matrix before it is embedded in the matrix and becomes an osteocyte (Owen, 1963). The transition stage between osteoblast and osteocyte in embryonic chick bone has been termed the osteoid-

osteocyte (Palumbo, 1986). This cell type is still capable of synthesising matrix proteins and is completely surrounded by osteoid.

b) *In vitro*

The heterogeneity of bone tissue poses problems concerning the interpretation of results. A method to simplify any system is to break it down into its component parts and to examine these individually. The culture of osteoblastic cells can be used to answer questions concerning, for example, the regulation and mechanisms of osteoblastic behaviour. The results from such studies, however, may not bear a direct correlation with the *in vivo* situation, although they will be an approximation and will aid the interpretation of *in vivo* data.

In vitro, osteoblasts can be studied in organ culture, primary cell culture or as a clonal cell line. The simplest technique used to study bone tissue *in vitro* has been organ culture and the most common tissue source used is fetal rat calvaria, due to the large size and accessibility of these bones. Fetal rat tibiae have also been used (Stracke *et al.*, 1984). Calvaria are dissected out from fetal rats and then split along the saggital suture to give two segments each consisting of a frontal and parietal bone (Figure 3) (Dietrich *et al.*, 1976). The calvarial halves are then incubated in liquid medium for up to 48 hours at 37°C. This method is also successful if neonatal rats are used as the source of calvarial tissue (Jones and Boyde, 1976). Organ culture need not necessarily be of the whole bone. Portions of calvaria can be excised in a punch biopsy and then cultured. A segment 5 to 8mm in diameter is taken from the the frontal calvarial bones of neonatal rats and then incubated in liquid medium at 37°C (Hahn *et al.*, 1984; Ituarte *et al.*, 1988).

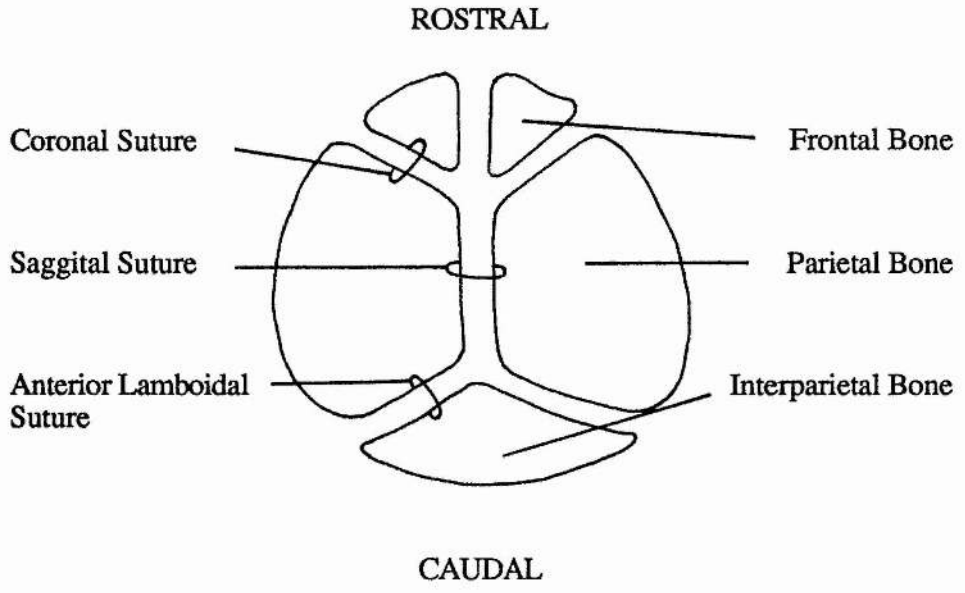


Figure 3. Diagram describing the anatomy of the calvarial plates.

The use of organ culture provides a system which maintains the inter-cell spatial arrangements and contacts and as such is very like the *in vivo* situation. However, these methods provide scant information concerning the function and responses of individual cell types. To do this it is necessary to isolate the bone cell types and study cultures derived from purified cell fractions. There are various methods for doing this and they can be broadly divided into mechanical and enzymatic.

An early mechanical method involved cell dispersal from adult rat calvaria by shaking for 45 minutes in phosphate buffer (Smith *et al.*, 1973). Osteoblasts have also been isolated by using their ability to migrate (Jones and Boyde, 1977). Fragments of parietal bone from 2 week old rats were incubated in contact with glass spicules for up to 5 days, during which time cells migrated from the bone onto the glass. This method was later refined, using more immature cells from periosteum-free neonatal mouse calvaria and allowing the cells to migrate directly onto the Petri dish surface (Zambonin Zallone and Teti, 1984). The cells growing out from the fragments are alkaline phosphatase positive, indicating the osteogenic nature of the migratory cells (Takahashi *et al.*, 1986). Cultures established by calvarial outgrowth are slow to produce a high cell yield and may even need

to be passaged before being used, which could result in non-mitotic osteoblasts being over-run by more prolific cell types, eg fibroblasts.

Enzymatic isolation techniques produce a high yield of primary cells and can be used to establish cultures derived from cell populations with different properties eg response to parathyroid hormone and calcitonin. Peck and colleagues (1964) prepared a single cell suspension of bone cells by incubating fetal and neonatal rat calvaria with collagenase. All the cells released exhibited detectable alkaline phosphatase and half showed intense cytoplasmic reaction. Other enzymes were also tried but these caused either too much cell damage (pronase) or released too few cells (trypsin). Another early attempt to isolate bone cells used a cocktail of enzymes (Hekkelman and Moskalewski, 1969). Collagenase, trypsin and DNA-ase dissolved in phosphate buffered saline were found to release alkaline phosphatase positive cells from fetal rat calvaria. Tryspin alone has also been used on periosteum-free fetal rat calvaria (Binderman *et al.*, 1974). Alkaline phosphatase activity was found in the bone cells isolated, and cultures established with these cells produced a calcified extracellular matrix.

A major step forward in the production of bone cell suspensions was the development of sequential digestion by Wong and Cohn (1974). They digested neonatal mouse calvaria with an enzyme solution of trypsin and collagenase for a total of one hour. However, at twenty minute intervals the enzymes were aspirated and replaced with fresh solution. This method produced three different aspirates, each containing a different population of bone cells. These populations were then characterised in terms of cAMP response to parathyroid hormone and/or calcitonin. The last population was the most responsive and also exhibited an additive effect of the hormones, thus showing;

i) the existence of a sub-population of bone cells acting as a target for parathyroid hormone and calcitonin, and also

ii) the possibility of two cell types within this sub-population with different hormonal responses.

Cultures established following five digestions showed the early populations (2 and 3) to respond to calcitonin and the late populations (3, 4 and 5) to respond to parathyroid hormone (Wong and Cohn, 1975). Populations of bone cells with different properties can therefore be isolated from bone using sequential digestion.

The methodology of sequential digestion has the advantage of producing functionally different bone cell populations. However, large numbers (> 50) of calvaria are required to achieve a good cell yield (Boonekamp *et al.*, 1981). Careful dissection of the bones before digestion can greatly reduce contamination by non-osteoblastic cells. Yagiela and Woodbury (1977) dissected out the frontal and parietal bones of fetal and neonatal rats, ensuring that the bone segments were free of both suture tissue and immature bone. The periosteum was then peeled off the bones and the fragments incubated with Worthington collagenase class II for two hours. They found that the highest numbers of osteoblastic cells (as judged by morphological criteria) were present in the 18 to 19 day old rat fetus. Cells with osteoblastic properties can also be isolated from fetal chick calvaria using a similar method (Nijweide *et al.*, 1981).

Contamination by non-osteoblastic cell types can be avoided, not only by careful dissection, but also by discarding the supernatant of early digests (Heath *et al.*, 1984). Neonatal mouse calvaria were stripped of periostea and sutures and then incubated with trypsin (10 minutes) and dispase (30 minutes). The cell suspensions produced were discarded and a final two incubations with Worthington collagenase class II (30 minutes each) were pooled. The cell population was enriched for osteoblasts, as assessed by alkaline phosphatase content, collagen type I synthesis and cAMP response to parathyroid hormone.

It is possible, therefore, to establish cultures of osteoblastic cells from the relatively heterogeneous tissue source of fetal and neonatal bone. To purify further osteoblastic cultures it is possible to produce cloned cell lines; at least two such exist. ROS cell lines were established by digesting minced rat osteosarcoma tissue with hyaluronidase and collagenase for 20 minutes (Majeska *et al.*, 1978). Trypsinised cultures were diluted and seeded into multiwell plates to encourage clonal development. The ROS lines exhibited a

pronounced cAMP response to parathyroid hormone and no response to calcitonin and they also retained an osteogenic ability, forming osteogenic tumours following transplantation *in vivo*. MC3T3 lines were established from neonatal mouse calvaria (Kodama *et al.*, 1981). Primary cultures were produced by allowing cellular outgrowth from periosteum-free bone fragments *in vitro*. The cells were passaged using trypsin and selection of a suitable clone, MC3T3-E1, was based upon alkaline phosphatase activity.

ii) Osteoclasts

a) *In vivo*

The osteoclast is a large multinucleated cell found associated with bone surfaces and it is the major cell type involved in bone resorption (Marks and Popoff, 1988). The cell membrane of the active osteoclast in contact with the bone is divided into two functional areas. The inner, central area of the membrane forms a ruffled border and is the site of secretion of enzymes. The extracellular space bounded by the ruffled membrane is also acidified due to the catalytic action of carbonic anhydrase on metabolic products (Hall and Kenny, 1986);

$$CO_2 + H_2O \rightarrow H^+ + HCO_3^-$$

The outer ring of membrane is flattened and is in close contact with the bone surface to form a clear zone functioning as a tight lateral seal limiting the area of bone resorption and thus allowing the formation of a microenvironment suitable for osteolysis (Sakamoto and Sakamoto, 1986). Inactive osteoclasts are not in contact with the bone surface and are kept away by the covering of osteoblasts and bone lining cells (Sterrett, 1986). Inactive osteoclasts do not possess a ruffled membrane.

Osteoclasts are possibly the end cell derived from a stem cell located in the marrow. However, it was once believed that the osteoclast was intimately connected with the osteogenic lineage (Hall, 1975). Evidence for this was seen in the co-ordinated decrease in osteoclasts and increase in osteoblasts and preosteoblasts *in vivo* following administration of calcitonin. This does not, however, prove that the former can develop into the latter. It is possible, however, that multinucleated osteoclasts are formed by the fusion of a variety

of cell types; local mononucleated preosteoclasts, osteocytes, preosteoblasts and blood-borne cells (Hanaoka *et al.*, 1989).

The distinctness of osteoclasts from the osteogenic lineage has been shown by studying the quail nuclear marker during endochondral ossification (Kahn and Simmons, 1975; Jotereau and Le Douarin, 1978). On the basis that chick chromatin is evenly distributed and quail chromatin is found in clumps it is possible to follow cell lineages in chimeric bones. Transplantation of quail limb bud onto chick embryo chorio-allantoic membrane resulted in endochondral ossification of the transplanted tissue and the formation of a bone organ with associated marrow. All the stroma cells were of donor (quail) origin and all the haemopoietic cells of host (chick) origin. It was also found that none of the osteoclasts exhibited quail chromatin, ie they were of host origin, indicating a blood borne (as opposed to osteal) and therefore haemopoietic derivation. Transplantation from chick to quail produced similar results, though not as conclusively as only two thirds of quail cells possess clumped chromatin.

The preosteoclast is a mononuclear precursor with the morphology of a mononuclear leukocyte (Bonucci, 1981). Osteoclasts have been shown to develop in initially osteoclast-free explants of fetal mouse calvaria following co-culture with previously cultured mononuclear cells from adult marrow (Ko and Bernard, 1981). Osteoclasts were not found in control cultures, showing that the osteoclastic precursors were amongst the mononuclear population.

The mononuclear nature of preosteoclasts has also been shown by using monoclonal antibodies raised against cells of the monocyte-macrophage lineage (Sminia and Dijkstra, 1986). In fetal rats specific antibody-positive cells were found in the perichondrium and were mononucleate and acid phosphatase positive. The expression of acid phosphatase is characteristic of the osteoclastic phenotype (Bonucci, 1981). The antibody positive cells then infiltrated the developing bone and multinucleated antibody positive cells were also seen within the marrow cavity. As the antibody reacts with antigens found solely on mononuclear phagocytes, the multinucleated acid phosphatase positive cells associated with the developing bone are also members of this series.

As has been seen, the haemopoietic and osteogenic lineages in the adult animal self-maintain independently (Friedenstein and Kuralesova, 1971). Therefore the osteoclast is ultimately derived from a haemopoietic stem cell (Schneider *et al.*, 1986). Developmentally, osteoclast stem cells and/or progenitors migrate from the yolk sac and are initially widely disseminated throughout the fetus (Thesingh, 1986).

The major role of the osteoclast is bone resorption. *In vivo*, resorption is followed by bone formation and the two complementary processes are coupled together to maintain a steady bone mass, despite variable turnover. This mechanism is referred to as coupling and it occurs during bone remodeling (Peck and Woods, 1988).

The remodeling cycle is characterised by the orderly progression through the sequence;

i) quiescence,

ii) initiation and activation,

iii) resorption,

iv) reversal,

v) formation,

vi) return to quiescence,

(Kahn *et al.*, 1983; Marcus, 1987; Martin *et al.*, 1988; Parfitt, 1988; Peck and Woods, 1988; Raisz, 1988).

During the quiescent stage the resting bone surface is covered in bone lining cells or resting osteoblasts. This is the normal state of the majority of the adult bone surface. Protecting the bone is a very thin layer of unmineralised matrix which is a sufficient barrier to osteoclastic resorption (Chambers and Fuller, 1985). However, if the surface is removed by collagenase digestion then osteoclastic resorption can proceed. Therefore, *in vivo*, the unmineralised matrix must be removed before osteoclast activation occurs. It is likely that the cell responsible for this is a member of the osteogenic lineage as pre-incubation of bone slices with calvarial cells greatly enhances the resorptive ability of osteoclasts. It has been shown that osteoblastic cells possess receptors for parathyroid hormone and respond to stimulation by this hormone by synthesising and secreting

collagenase (Heath *et al.*, 1984; Puzas and Brand, 1979; Silve *et al.*, 1982). The action of parathyroid hormone upon osteogenic cells results in the digestion of the unmineralised layer of matrix thus exposing the bone mineral. The release of parathyroid hormone may represent an initiation event causing the activation of osteoblasts and the subsequent chemotactic response of osteoclasts, possibly to the exposed bone mineral (Osdoby *et al.*, 1987). Monocytes can respond chemotactically to bone proteins (osteocalcin, a_2HS glycoprotein and collagen type I) (Malone *et al.*, 1982). Not only do hormones which stimulate bone resorption, for example parathyroid hormone and prostaglandin E_2, promote collagenase release, they also cause shape changes in osteoblastic cells, thus uncovering the matrix (Rodan and Martin, 1981). Osteoblasts may also play an important role in the differentiation of mononucleate osteoclast precursors by fusion to form functional osteoclasts (Takahashi *et al.*, 1988).

Following the activation phase of remodeling the bone mineral is resorbed by osteoclasts, forming Howship's lacunae in trabecular bone. Bone resorption is dependent upon osteoclastic acid phosphatase, as inhibition of this enzyme by specific antibodies results in the loss of resorption (Zaidi *et al.*, 1989). According to the the two cell theory of bone resorption more than one cell type is involved in the removal of matrix (Heersche, 1978; Sterrett, 1986). Osteoclasts demineralise and then degrade the non-collagenous matrix, but the removal of collagen fibrils may be the function of mononuclear phagocytes. This was proposed to explain the absence of collagen fibres within osteoclasts. However, this could also be explained by the hypothetical existence of collagen as a collagen-collagenase-collagenase inhibitor complex. Inactivation of the inhibitor by osteoclastic action would allow the degradation of collagen by collagenase in the extracellular space, rather than by breakdown in the osteoclast cytoplasm (Sakamoto and Sakamoto, 1986). In support of this, osteoclastic degradation of collagen has been shown to be independent of the secretion of collagenase (Blair *et al.*, 1986). The control of osteoclastic resorption during steady state turnover is unknown but it continues over a period of one to three weeks to a mean depth of 50μm. Osteoclasts are then replaced by mononuclear cells, possibly of the monocyte-macrophage lineage, which smooth over the surface of the pit,

possibly by removing the exposed collagen fibres. These cells may also deposit the cement line, demarcating the furthest extent of resorption. The stage between the end of resorption and the beginning of bone formation is known as reversal and may last for one to two weeks.

Bone formation is initiated by the recruitment of osteoblasts and the subsequent secretion of matrix. Osteoblasts *in vitro* respond chemotactically to a protein component of bone matrix (Somerman *et al.*, 1983). Resorbing bone is also a good source of mitogenic activity which may act on osteoprogenitor cells to produce an increase in osteoblast number (Farley *et al.*, 1987). The attraction of osteoblasts to the site of bone remodeling may therefore be controlled by the release of chemoattractants and mitogens from secondary lysosomes of osteoclasts following resorption from the matrix.

Initially, the matrix formed by the osteoblast is unmineralised but as formation progresses the matrix calcifies and a mineralisation front forms, above which lies an osteoid seam. The most recently formed collagen fibres in the osteoid are disorganised but become increasingly aligned due to cross-linking before the matrix is mineralised. As formation progresses the osteoblast becomes smaller and there is a probable corresponding decrease in the rate of matrix synthesis. Eventually the osteoid seam disappears as the mineralisation front advances and the bone surface returns to the quiescent state.

b) *In vitro*

Osteoclastic behaviour *in vitro* can be studied in organ culture or as isolated cells in primary culture. Organ culture is used to study the mechanisms and control of resorption. Typically, rats at day 18 of pregnancy are injected with a radioactive isotope of calcium, ^{45}Ca (Ibbotson *et al.*, 1986; Raisz and Niemann, 1969). The following day, the fetuses are removed and the shafts of the radii and ulnae dissected out and cultured in liquid medium for up to five days. The amount of ^{45}Ca released is used as a measure of bone resorption. A similar method involves neonatal mouse calvaria (Reynolds, 1976). The mice are prelabelled with ^{45}Ca and then half calvaria are cultured as explants at the liquid/gas interface for two days.

Cultures of isolated osteoclasts can give more information concerning, for instance, the specific cellular response to bone hormones. To establish such cultures, the femoral and tibial shafts of neonatal rats are split longitudinally and the cut surface is scraped to release bone fragments into culture medium (Chambers and Magnus, 1982). The fragments are agitated by pipetting to release cells and aliquots of the medium transferred to tissue culture grade Petri dishes. Following a 30 minute incubation at 37°C, the dishes are washed and the medium replaced. The adherent cells remaining in the Petri dishes are predominantly osteoclasts; they are large multinucleated cells with a membranous 'skirt' giving a 'fried egg' appearance (Arnett and Dempster, 1986; Chambers and Magnus, 1982).

The regulation of osteoclastic behaviour is complex and not always direct. Two potent stimulators of bone resorption are parathyroid hormone (PTH) and a metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). *In vivo*, a drop in blood calcium levels stimulates the parathyroid gland to release PTH (Dickson, 1987). The hormone can then influence bone resorption via two prominent mechanisms.

PTH can act directly upon bone cells (Chambers *et al.*, 1984). Added to isolated osteoclasts, PTH has no discernible effect, however, co-culture of osteoclasts with osteoblasts results in increased osteoclastic spreading and bone resorption (McSheehy and Chambers, 1986a). PTH stimulates the osteoblastic cells to produce a factor that directly affects osteoclasts and stimulates bone resorption (McSheehy and Chambers, 1986b). PTH has also been shown to stimulate osteoblastic cells to secrete prostaglandin E (MacDonald *et al.*, 1984). Prostaglandins can either inhibit osteoclast motility, or can stimulate the release of osteoclast activation factor (OAF) from phytohemaglutin-stimulated lymphocytes (Chambers and Ali, 1983; Sterrett, 1986).

Alternatively, PTH can influence the production of $1,25(OH)_2D_3$ (Dickson, 1987). Vitamin D is converted to 25-hydroxyvitamin D_3 in the liver. This metabolite is acted upon by a PTH-regulated kidney hydroxylase to produce $1,25(OH)_2D_3$. The systemic effect of $1,25(OH)_2D_3$ is to increase blood calcium levels by;

i) stimulating the synthesis of calcium-binding protein by the gut and thus calcium absorption, and

ii) stimulating bone resorption.

In bone, the cellular site of action of $1,25(OH)_2D_3$ is very likely to be the osteoblast. Two cell lines have been shown to possess cytosolic receptors for $1,25(OH)_2D_3$ (Partridge et al., 1980; Walters *et al.*, 1982). The indirect effect of $1,25(OH)_2D_3$ upon bone resorption may be mediated through osteoblastic cells, in a manner similar to that of PTH.

Vitamin D is also, paradoxically, involved in mineralisation (Wong *et al.*, 1977). However, there are many metabolites of this vitamin and it is probably the concerted action of $1,25(OH)_2D_3$ raising the serum calcium levels (by increased absorption from the intestine) with 24,25-dihydroxyvitamin D_3 that enhances mineralisation (Ornoy *et al.*, 1978).

Calcitonin, produced in the thyroid, acts directly via an osteoclastic receptor to cause inactivation and inhibition of bone resorption (Nicholson *et al.*, 1986). Exposure of isolated osteoclasts to calcitonin results in a decrease in motility and retraction of lamellipodia (Chambers and Magnus, 1982). The transformation caused by calcitonin occurs at very low concentrations (> 3pg/ml), is reversible and can be prevented by pre-incubation with trypsin (Chambers and Moore, 1983).

In summary, bone is very much a live tissue exhibiting continual and controlled turnover of the extracellular matrix by osteoclasts and osteoblasts. Terminal differentiation of osteoblasts to osteocytes may play an important role in calcium homeostasis, as may hormonal control of resorption. The two main cell types associated with bone are osteoblasts and osteoclasts and these are derived from stem cells, respectively stromal and haemopoietic, and these are located within the marrow cavity.

2. The extracellular matrix

The extracellular matrix of bone consists of a mineralised framework mainly of type I collagen, and some non-collagenous proteins. The organic component is synthesised and secreted largely by osteoblasts but it does contain some plasma-derived proteins. The matrix is mineralised by the deposition of hydroxyapatite (a form of calcium phosphate). The non-collagenous proteins are probably involved in the general maintenance and regulation of the matrix secretion and mineralisation (Table 1.)

Molecule	Mass	Amount	Origin	Known (and possible) roles
Collagen		90%[2]	osteoblasts	structural[6] (mitogen[8])
Osteonectin	32kD[11]	2.5%[9]	osteoblasts	collagen-apatite bridge[2] mineralisation regulator[1] (cell-substrate bridge[10])
α_2HS-glycoprotein	50kD[12]	2.5%[2,9]	liver cells[12]	binds to mineral[12] (role in resorption[12])
Osteocalcin	6kD[12]	2.0%[2]	osteoblasts[12]	chemoattractant for osteogenic cells binds mineral[7]
Proteoglycans	78kD - 120kD[2,3]	1.0%[2]	?	(aid collagen fibril assembly[12])
Bone sialoprotein	23kD[12]	0.8 - 1.2%[12]	?	cell adhesion[5] binds to calcium[13]

Table 1. Summary of the main molecules associated with the bone matrix

1. Doi *et al.*, 1989
2. Gehron Robey *et al.*, 1988
3. Goldberg *et al.*, 1988
4. Lucas *et al.*, 1988
5. Oldberg *et al.*, 1986
6. Piez, 1987
7. Price, 1983
8. Rath and Reddi, 1979
9. Rodan and Rodan, 1984
10. Stenner *et al.*, 1986
11. Termine *et al.*, 1981
12. Triffitt, 1987
13. Vaughan, 1984

i) The organic extracellular matrix

a) Collagen

Type I collagen is the major constituent of bone matrix, of which it comprises roughly 90% of the organic part (Rodan and Rodan, 1984). Collagen is formed from three collagenous propeptide chains wound round each other to produce a triple helix structure (Gehron Robey *et al.*, 1988). The polypeptide chains of type I collagen are two α1(I) chains and one α2(I) chain. There are at least ten different collagen types whose functions are predominantly structural (Piez, 1987). It may also act as a local mitogen (Rath and Reddi, 1979).

Triple helical procollagen is released from secretory vesicles into the extracellular space where the molecule is converted to collagen by the enzymatic removal of the amino- and carboxyl-terminal propeptides. The collagen molecules are then arranged into bundles to form collagen fibrils which are characterised by a regular banding pattern. Cross-linking then occurs between the fibrils to produce a very stable structure and to protect the molecule from degradation (Laurent, 1987). Nevertheless, collagen molecules undergo degradation both intra- and extracellularly, possibly to preserve collagen homeostasis.

The secretion of normal collagen is dependent upon the presence of ascorbic acid (de Clerck and Jones, 1980). It is necessary for the normal synthesis of hydroxyproline (which stabilises the helical structure) and hydroxylysine (which forms the cross-links) (Murad *et al.*, 1981). Ascorbic acid functions as a co-factor in the hydroxylation of prolyl and lysyl residues (Pinnell *et al.*, 1987). Ascorbic acid has also been shown to cause an increase in the levels of mRNA for the procollagen polypeptide chains.

Collagen fibrils within adult cortical bone assume a very organised architecture (Giraud-Guille, 1988). The arrangement can resemble either 'twisted plywood' or 'orthogonal plywood'. The collagen fibrils of orthogonal plywood form distinct layers, each lying at right angles to those adjacent, forming orientated collagen sheets. Twisted plywood structure appears as a series of nested arcs producing a scalloped pattern. It is

likely that the development of the fibril orientation is controlled by osteoblasts (Jones and Boyde, 1976).

b) Glycoproteins

The bone matrix contains several different glycoproteins. Bone sialoprotein accounts for 8% to 12% of the non-collagenous protein (Triffitt, 1987). Bone sialoprotein contains roughly 20% sialic acid (Butler, 1987). Two other proteins, sialoprotein I and sialoprotein II, contain 5% and 13% sialic acid respectively. Sialoprotein I (or osteopontin) and sialoprotein II share sequences with fibronectin and vitronectin and can cause the adhesion and spreading of ROS cells (Oldberg *et al.*, 1986; Oldberg *et al.*, 1988b). Indeed, the receptor for osteopontin is identical to that for vitronectin. Sialoprotein mRNA is found in bone, but not in ROS cells, suggesting that osteoblasts may not necessarily be the source of this glycoprotein (Oldberg *et al.*, 1988a). Despite the marked presence of sialoproteins within the bone matrix, their function is unknown. Apart from the cell-binding regions they exhibit strong calcium binding properties and may act as organic-inorganic bridges (Vaughan, 1984).

In fetal calf bone, approximately 25% of the non-collagenous protein is osteonectin (Rodan and Rodan, 1984). The abundance of this glycoprotein indicates an important bone-related function. Osteonectin has been found to contain covalently bound phosphate groups and to possess binding sites for collagen and hydroxyapatite. It may act as a bridge between these two matrix components, possibly functioning as a nucleator of mineralisation (Termine *et al.*, 1981; Termine, 1983; Triffitt, 1987). Although osteonectin has been shown to enhance apatite binding to collagen at 25°C, it inhibits the formation of hydroxyapatite crystals at 37°C (Doi *et al.*, 1989). Osteonectin may act by preventing disorderly precipitation from super-saturated solutions. The distribution of osteonectin is limited to mineralising bone trabeculae and consequently it is a tissue-specific protein. Immunocytochemistry using polyclonal antibodies raised against osteonectin shows it to be a marker of osteogenic differentiation (Jundt *et al.*, 1987). It is found in osteoprogenitor cells, osteoblasts and immature osteocytes.

Osteonectin is synthesised *in vitro* by osteoblastic cells isolated by enzymatic digestion of fetal porcine calvaria. It is also found within platelets and may be released during coagulation. This possibility has given rise to the idea that osteonectin may also be involved in the adhesion of cells to substrates (Otsuka *et al.*, 1984; Stenner *et al.*, 1986; Tracy *et al.*, 1988). Osteonectin shows some sequence homology with two glycoproteins not associated with bone, namely a 43K protein and SPARC (secreted protein which is acidic and rich in cysteine) (Tracy *et al.*, 1988).

Thrombospondin is an adhesive glycoprotein synthesised by osteoblasts and is incorporated into the matrix, although this may not be the sole source of bone thrombospondin (Gehron Robey *et al.*, 1989). The molecule is endowed with several binding sites for Ca^{2+} as well as for collagen and has been found to form complexes with osteonectin; this may reflect its bone-related function (Clezardin *et al.*, 1988).

c) Gla-containing proteins

Two γ-carboxyglutamic acid (Gla)-containing proteins are found in the bone matrix. The presence of Gla in clotting factors is important during coagulation (Triffitt, 1987). Glutamic acid is converted to Gla by a vitamin K-dependent process.

Bone Gla protein (BGP) or osteocalcin is a highly conserved bone protein which accounts for about 20% of non-collagenous protein (Gehron Robey *et al.*, 1988; Groot *et al.*, 1986). During induced endochondral osteogenesis the appearance of osteocalcin coincides with the calcification of the matrix (Hauschka and Reddi, 1980). It is found in the mineralised matrix, but not in the osteoid, although this may be an artifact (Groot *et al.*, 1986). The function of osteocalcin *in vivo* is unknown, but it may act as a nucleator of mineralisation due to its high affinity for hydroxyapatite and its ability to interact with the phospholipids of the matrix vesicle membrane (Gendreau *et al.*, 1989; Price, 1983). Osteocalcin also acts as a potent chemoattractant for ROS cells (a cell line derived from a rat osteosarcoma), chick limb bud mesenchymal cells and chick embryonic fibroblasts (Lucas *et al.*, 1988; Mundy and Poser, 1983). During remodelling osteocalcin will be released as the matrix is degraded and may therefore be able to act as a chemoattractant for

osteogenic cells. It is likely that osteocalcin is synthesised and secreted by a cell belonging to the osteogenic lineage. Its production is stimulated by $1,25(OH)_2D_3$ and inhibited by TGFß (Lian *et al.*, 1985; Noda, 1989; Price, 1983). This response to $1,25(OH)_2D_3$ is very specific and provides a method to identify osteoblasts *in vivo* (Gallagher *et al.*, 1986).

As the synthesis of osteocalcin is dependent upon vitamin K, the function of osteocalcin can be studied in animals treated with the vitamin K antagonist, Warfarin (Price, 1983). However, Warfarin-treated animals appear to have normally mineralised bones, however, although there are some abnormalities associated with the mineralisation of cartilage. These effects are due to the inhibition of synthesis of another Gla-containing protein, matrix Gla-protein (MGP) (Price, 1988).

MGP is found within the matrices of bone and cartilage, and in bone it is associated with BMP (Triffitt, 1987). It is a larger molecule than osteocalcin but they share homologous sequences, suggesting a common genetic ancestor.

d) Proteoglycans

Proteoglycans are characteristically composed of a protein core with glycosaminoglycan side chains. In mineralised matrix they constitute roughly 10% of the non-collagenous protein (Gehron Robey *et al.*, 1988). The function of proteoglycans may be to aid the assembly of collagen fibrils (Triffitt, 1987). Proteoglycans have been found to be localised to the developing bone matrix and osteogenic cells (Termine, 1983).

Bone proteoglycans are small for this class of molecule and consist of a protein core (molecular mass 38kD), off which lie either one (PGII, or decorin) or two (PGI, or biglycan) chondroitin sulphate chains (40kD each) and several oligosaccharides (Fisher *et al.*, 1989). PGI and PGII are immunologically distinct from each other (Prince, 1987). Similar proteoglycans are synthesised by osteoblasts *in vitro*, however, the chondroitin sulphate chains are replaced with dermatan sulphate (Ecarot-Charrier and Broekhuyse, 1987). Keratan sulphate proteoglycan (KSPG) is found in rabbit cortical bone and it has a core protein identical to sialoprotein (Kinne and Fisher, 1987). Three hydroxyapatite-

associated proteoglycans (HAPG1, 2 and 3) have been recorded (Goldberg *et al.*, 1988). HAPG1 has a molecular weight of 110 - 120kD and a protein core of 45kD. HAPG2 and HAPG3, though distinct, have a total weight of 100 - 110kD and protein cores of 37 - 38kD.

e) Plasma proteins

The bone matrix contains proteins concentrated from plasma. α_2HS glycoprotein accounts for approximately 25% of the non-collagenous protein (Rodan and Rodan, 1984). α_2HS glycoprotein is synthesised in the liver and has a high affinity for bone mineral and may play a role in resorption (Triffitt, 1987). Serum albumin is also found in bone (Gehron Robey *et al.*, 1988).

f) Growth factors

Growth factors are usually small polypeptides with hormone-like characteristics. They function as growth regulators by acting as mitogens (Canalis, 1988; Maclean and Hall, 1987). Growth factors associated with the bone matrix may play an important role in the coupling of resorption and formation as well as acting as mediators for systemic hormones. Growth factors found within the matrix include fibroblast growth factor (both acidic and basic), transforming growth factor ($ß_1$ and $ß_2$), insulin-like growth factor (I and II), bone-derived growth factor, platelet-derived growth factor and skeletal growth factor (Table 2) (Canalis, 1988; Lau *et al.*, 1988).

Transforming growth factor (TGF) can induce the formation of anchorage-independent colonies by non-neoplastic cells. There are two classes of TGF; α and ß. TGFα acting alone can induce colony formation, and it is not found in bone. TGFß induces colony formation in the presence of epidermal growth factor (EGF) and is found in three forms; $ß_1$, $ß_2$ and $ß_{1.2}$ (Canalis, 1988). TGFß is probably identical in structure and function to cartilage-inducing factor A (Ellingsworth *et al.*, 1986).

Bone TGFß is not a plasma-derived factor, but is synthesised and secreted by osteoblastic cells, although TGF$ß_1$ mRNA is also found in osteoclasts (Gehron Robey *et*

al., 1987; Sandberg *et al.*, 1988). During endochondral osteogenesis TGFß is localised within the developing mineralised matrix (Carrington *et al.*, 1988). *In vitro*, TGFß affects the differentiation and proliferation of bone cells. Cell growth is inhibited in two cell lines, ROS and MC3T3-E1, by TGFß in the range 2 to 10ng/ml (Elford *et al.*, 1987b; Noda and Rodan, 1986; Noda and Rodan, 1987). However, the phenotypic expression of alkaline phosphatase is stimulated in ROS cells and inhibited in MC3T3-E1 cells. Primary bone cell cultures show stimulated proliferation and collagen synthesis in response to TGFß and inhibition of alkaline phosphatase activity (Centrella *et al.*, 1987a; Centrella *et al.*, 1987b; Rosen *et al.*, 1988; Wrana *et al.*, 1988).

TGFß may act as a coupling factor during the cycle of resorption and formation. Osteoclasts are capable of synthesising TGFß, which is activated by an acidic environment, such as that found associated with the ruffled border of activated osteoclasts. The activated TGFß could then act upon osteoblastic cells consequently stimulating both collagen synthesis and proliferation in direct relation to osteoclast action.

Bone-derived growth factor (BDGF) is synthesised, though not exclusively, by bone cells (Canalis, 1988; Canalis *et al.*, 1988b). However, to call BDGF a growth factor may be a misnomer, as it is $ß_2$ microglobulin, a small globular peptide associated with a major histocompatability complex antigen. Nevertheless, BDGF has been found to stimulate collagen and DNA synthesis in bone, as well as to act as a mitogen for fibroblasts (Centrella and Canalis, 1985; Canalis and Centrella, 1986). Six other distinct BDGFs have been extracted from fetal calf bone (Hauschka *et al.*, 1986).

Insulin-like growth factor I (IGF I), also known as somatomedin-C, is synthesised by bone cells in organ culture and by a bone cell line, UMR (Canalis *et al.*, 1988c; Gray *et al.*, 1989). IGF II is also found in the bone matrix but it is not known whether it is synthesised by bone cells (Canalis *et al.*, 1988b). IGF I stimulates collagen and matrix synthesis, alkaline phosphatase activity, cell replication, and provides a local mechanism for the action of growth hormone (Ernst and Froesch, 1988; Hock *et al.*, 1988; McCarthy *et al.*, 1989a; Schmid *et al.*, 1984). Synthesis of IGF I by bone cells is stimulated by PTH (McCarthy *et al.*, 1989b).

Growth factor	Mass	Origin	Action on osteoblasts	
TGFß	25kD[3]	osteoblast[16,12] osteoclast[24]	proliferation;	increase[6,7,12] decrease[8,15,21,22]
			alkaline phosphatase;	increase[22] decrease[7,8,15,21]
			collagen synthesis;	increase[7,11,22] decrease[15]
			cAMP resp to PTH;	decrease[8,15,16]
SGF	83kD[10]		proliferation;	increase[10,18]
IGF I	7.5kD[4]	osteoblast[14]	proliferation;	increase[4,17]
			collagen synthesis;	increase[4,17,19]
			IGF I synthesis increased by;	PTH[20], $1,25(OH)_2D_3$[14], estradiol[14], growth hormone[9]
PDGF	27-30kD[3]		proliferation;	increase[1]
			collagen synthesis;	decrease[1]
BDGF	11kD[5]		proliferation;	increase[5]
			collagen synthesis;	increase[5]
			alkaline phosphatase;	decrease[23]
aFGF	16kD[3]	osteoblast[13]	proliferation;	increase[12, 13, 23]
			alkaline phosphatase;	decrease[23]
bFGF	17kD[3]	osteoblast[13]	proliferation;	increase[2,12,13]
			collagen synthesis;	decrease[2]

Table 2. Summary of the characteristics of the main growth factors found within the bone matrix.

1. Canalis, 1981
2. Canalis *et al.*, 1988a
3. Canalis *et al.*, 1988b
4. Canalis *et al.*, 1988c
5. Canalis and Centrella, 1986
6. Centrella *et al.*, 1987a
7. Centrella *et al.*, 1988
8. Elford *et al.*, 1987
9. Ernst and Froesch, 1988
10. Farley and Baylink, 1982
11. Gehron Robey *et al.*, 1987
12. Globus *et al.*, 1988
13. Globus *et al.*, 1989
14. Gray *et al.*, 1989
15. Guenther *et al.*, 1988
16. Gutierrez *et al.*, 1987
17. Hock *et al.*, 1988
18. Lau *et al.*, 1988
19. McCarthy *et al.*, 1989a
20. McCarthy *et al.*, 1989b
21. Noda and Rodan, 1986
22. Noda and Rodan, 1987
23. Rodan *et al.*, 1987
24. Sandberg *et al.*, 1988

Platelet-derived growth factor (PDGF) is synthesised by bone cells and is found in the matrix (Canalis, 1988; Canalis *et al.*, 1988b). PDGF has been shown to stimulate proliferation and protein synthesis. However, it can also stimulate resorption via a prostaglandin-dependent pathway. Acidic and basic fibroblast growth factors (aFGF and bFGF) are both synthesised by osteoblastic cells and are found in the bone matrix (Globus *et al.*, 1989). They both stimulate bone cell proliferation causing a subsequent increase in collagen synthesis. Skeletal growth factor (SGF) is also found in bone matrix (Farley and Baylink, 1982). SGF has been shown to stimulate proliferation in primary cultures of chicken bone cells, however, no such stimulation occurs in the MC3T3-E1 cell line. MC3T3-E1 cells respond to SGF with an increase in collagen synthesis (Lau *et al.*, 1988; Linkhart *et al.*, 1986). The primary bone cells may represent a more immature cell type and SGF may, therefore, cause different effects in different osteogenic populations.

Bone morphogenetic protein (BMP) isolated from demineralised bone is capable of inducing osteogenic differentiation in connective tissue cells and was thought to be a glycoprotein (Urist *et al.*, 1979). However, BMP has been found to be composed of the combined action of several factors (Wozney *et al.*, 1988). At least three components of BMP are related to TGFß in sequence structure and another may act as a protease activator for $TGF\beta_1$.

ii) The inorganic extracellular matrix

a) The mineral phase

Bone mineral consists of calcium phosphate crystals. The mineral is probably deposited as octa-calcium phosphate ($Ca_8H_2(PO_4)_6$) but the mature form is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Mann, 1988). The mineral may also contain some carbonate (Gehron Robey *et al.*, 1988).

b) Mineralisation mechanisms

Mineralisation is initiated by the nucleating action of collagen fibrils or matrix vesicles, depending on the type of bone.

Woven bone (primary or immature) is found in fetal tissues and in areas of rapid bone growth. It is covered by a thin layer of osteoid and the collagen fibrils are randomly orientated. Mineral crystals first appear in association with matrix vesicles. These are membrane-bound extracellular vesicles containing glycoproteins, lipids, alkaline phosphatase and mineral ions, particularly Ca^{2+} and P_i (McLean *et al.*, 1987). The vesicles are probably derived by 'blebbing' from the cell membrane (Anderson, 1989).

Electron microscopy has shown that the earliest crystals form within matrix vesicles. As the crystals grow the vesicle is obliterated and the surrounding matrix is calcified (Bonucci, 1987). The role of alkaline phosphatase during crystal formation is unclear. Matrix vesicles do not always require alkaline phosphatase substrates to initiate mineralisation (Wuthier, 1986). It may be the Ca^{2+}-binding properties of alkaline phosphatase that are important during mineralisation (de Bernard *et al.*, 1986).

Lamellar bone (secondary or mature) is usually found in adult tissues. It is covered by a thick layer of osteoid and is characterised by the arrangement of the collagen fibrils into regular lamellae (Gehron Robey *et al.*, 1988). Very few matrix vesicles are found in lamellar bone and calcification is first seen in association with the collagen fibrils. As mineralisation progresses, the banding pattern is obliterated as the volume of crystal expands. The packing arrangement of collagen results in the formation of regular holes and grooves along the fibre (Mann, 1988). These spaces act as nucleators for the deposition of calcium phosphate. The hole zones may function as nucleators by providing the necessary environment for non-collagenous proteins to act as bridges between the collagen and mineral. It is possible that these molecules encourage mineral deposition by electrostatic accumulation of Ca^{2+} and phosphate ions. The local organisation of the ionic charge could result in the alignment of crystals parallel to the long axis of the collagen fibre.

The type of nucleating mechanism is dependent upon the behaviour of the osteoblastic cells associated with the bone. In woven bone, the osteoblasts produce a

matrix of randomly orientated collagen interspersed with matrix vesicles. The matrix vesicles serve as the initiators of calcification and it is possible that the disorganisation of collagen precludes any nucleating capability. The differences between lamellar and woven bone may be due to either the presence of different osteogenic cells, although members of the same lineage, or the existence of differential behaviour of the osteogenic cells in response to local and/or systemic control.

C. BONE FORMATION

The formation of bone *in vivo* is, normally, a very controlled process both spatially and temporally. Osteogenesis occurs initially during skeletal development and then continues during growth. Osteogenesis also takes place in the adult animal as part of the normal remodelling process.

1. Developmental bone formation

There are two methods of developmental bone formation;

i) endochondral osteogenesis, characterised by the formation of a cartilage model and its subsequent replacement with bone, and

ii) intramembranous osteogenesis, characterised by the direct transition of mesenchyme to osteogenic cells without chondrogenic involvement.

i) Endochondral osteogenesis

Embryonic bone is formed from mesenchymal cells derived from the mesoderm. The initial formation of cartilage cells by the differentiation of mesenchyme is induced by short-range (matrix-mediated) interactions with surrounding epithelia (Hall, 1988). The cartilage model is then replaced by bone cells possibly derived by;

i) direct transformation of the cartilage cells,

ii) inductive action by the cartilage on the surrounding cells,

iii) blood-borne migration from elsewhere in the embryo.

The appearance of osteoblasts precedes that of osteoclasts, possibly due to a chemoattractic response by osteoclast progenitor cells to osteoblastic products eg collagen and osteocalcin (Parry, 1985).

Mineralisation first occurs in the collar surrounding the middle of the shaft forming a ring of bone around a core of calcified cartilage. The shaft is then invaded by capillaries carrying in haemopoietic cells as well as undifferentiated mesenchymal cells from the perichondrium (Vaughan, 1981). The calcified cartilage core is then resorbed and replaced by bony trabeculae. Vascular invasion of the cartilaginous ends (epiphyses) occurs separately resulting in the formation of the epiphyseal growth plate, thus allowing longitudinal growth (Reddi, 1985).

It has also been suggested that instead of the cartilage rudiment forming a model for the bone, it may provide a model for the marrow cavity (Caplan, 1987; Caplan and Pechak, 1987). All bone formation occurs outside the first ring of diaphyseal bone while the cartilage model is replaced by marrow and vasculature.

Endochondral osteogenesis occurs during the development of the long bones, backbone, ribs and parts of the facial skeleton. Bone formed in this manner mineralises more rapidly than that formed by intramembranous osteogenesis (Dziedzic-Goclawska *et al.*, 1988). This may reflect the functional differences between weight bearing and non-weight bearing bones.

ii) Intramembranous osteogenesis

Osteogenesis is initiated by the differentiation of mesenchymal cells into osteoblasts (Marvaso and Bernard, 1977). These cells then synthesise a calcifiable extracellular matrix possibly containing matrix vesicles. Nucleation sites within the matrix are rich in sulphur-containing components, possibly proteoglycans (Arsenault and Ottensmeyer, 1984). The sulphur components are then overlaid by calcium and phosphorous and discrete nodules of bone form containing hydroxyapatite crystals. The nodules eventually coalesce to form seams of woven bone surrounded by a layer of fibroblasts. This sequence of events can

occur in the absence of epithelial interactions, although they may be necessary at earlier stages.

Intramembranous osteogenesis occurs during the development of the skull, most of the facial skeleton and the clavicle.

2. Bone repair

Following a fracture the first response is acute inflammation bringing in phagocytic cells and systemic factors eg growth factors (Caplan, 1987). The fracture gap is then plugged by a blastema of mesenchymal cells, possibly derived from the pool of bone cells, or by chemoattraction from a blood-borne source, or from an inducible osteogenic reserve of perivascular undifferentiated connective tissue cells (Mikhailova and Pal'tsyn, 1986). The mesenchymal cells then differentiate into chondrocytes, possibly due to the action of a soluble factor present in bone. The chondrocytes hypertrophy and the cartilage mineralises before osteogenesis occurs, probably in a manner similar to that seen in endochondral osteogenesis.

3. Experimental bone formation

i) *In vivo* bone formation

Bone development can be induced following implantation of demineralised bone into extra-skeletal sites *in vivo* (Bernick *et al.*, 1989; Harakas, 1984; Muthukumaran and Reddi, 1985; Urist, 1965). Induced osteogenesis by HCl-demineralised bone is initiated by the infiltration of connective tissue macrophages, lymphocytes and fibroblasts and the implant becomes covered in vascularised connective tissue (Bernick *et al.*, 1989; Urist, 1965). Vascular channels are then enlarged by the action of acid-phosphatase positive cells prior to bone formation. Following osteogenesis the implant can provide a suitable microenvironment for the establishment of a marrow organ by invading host haemopoeitic cells (Harakas, 1984).

Mesenchymal differentiation may be dependent upon the amount of vascularisation, as low oxygen tension encourages chondrogenesis and high oxygen tension encourages

osteogenesis. Implants exhibiting chondrogenic differentiation and subsequent osteogenesis provide a model system for induced endochondral osteogenesis (Muthukumaran and Reddi, 1985).

There are two important events occurring here;

i) the initial attraction of mesenchymal cells and blood vessels,

ii) the subsequent induction of osteogenic differentiation.

The location of the implantation site affects the extent of bone formation. Bone and bone marrow yield the greatest amounts, followed by skeletal muscle, whereas testes, pancreas and ovary are inefficient osteoinductors (Harakas, 1984).

The inductive ability of demineralised bone is due to the presence of BMP (Urist *et al.*, 1979). Other osteogenic factors can be isolated from demineralised bone matrix, as well as cartilage-inducing factor (Muthukumaran and Reddi, 1985). The action of BMP can be potentiated by interleukin-1 (IL-1) (Mahy and Urist, 1988). IL-1 may act as a mitogen for osteoprogenitors or it may modulate the response to BMP.

Bone can also form *in vivo* following the intra-muscular transplantation of freshly isolated bone cells from fetal rat calvaria (Groot *et al.*, 1983; Moskalewski *et al.*, 1983). The cells start to synthesise collagen by day 2 or 3, forming islands of matrix surrounded by alkaline phosphatase positive cells. The first bone laid down is woven bone but by 1 to 2 months lamellar bone predominates. Very few osteoclasts are observed and the lamellar bone consequently contains cores of woven bone and marrow cavities do not form. The lack of osteoclasts may be due to the absence of an appropriate chemoattractant in the transplant.

Bone cells from neonatal mouse calvaria implanted peritoneally in Millipore diffusion chambers also form bone (Simmons *et al.*, 1982). Following 20 to 30 days *in vivo* the chambers are found to contain a mineralised bone matrix and alkaline phosphatase positive cells. The matrix and cellular composition and the time scale is similar to that for osteoblastic cells implanted intra-muscularly without diffusion chambers. In this latter

case, it is as if the isolated osteoblastic cells are behaving as though they are within a sealed chamber, exhibiting no cellular interaction with the host.

There is, therefore, a very basic difference between bone formation following implantation of demineralised bone matrix and that following implantation of osteoblastic cells. Induction of bone formation involves the attraction, proliferation and differentiation of unspecialised mesenchymal cells to establish a bone organ from first principles. Implanted cells are obviously more differentiated initially and may have, in situ, passed through the stage capable of directly influencing mesenchymal cells.

ii) *In vitro* bone formation

a) Organ culture

Endo (1960) initially reported osteogenesis *in vitro* using organ culture. Embryonic chick femurs increased in length following culture in medium supplemented with chick embryo extract. However, abnormal calcification sequences were observed. Fetal rat radii and ulnae in organ culture are capable of collagen synthesis and both show increases in length (Raisz *et al.*, 1976). Collagen synthesis was stimulated by phosphate and ascorbic acid and inhibited by PTH. However, fetal rat calvaria, in terms of amount of tissue present, are far more amenable to organ culture.

In this system, the amount of collagen synthesised is used as a measure to enable the quantification of bone formation. Using this criterion, it has been shown that PTH, EGF and tumour necrosis factor inhibit bone formation and insulin, insulin-like growth factor (somatomedin) and cortisol all stimulate bone formation (Canalis, 1987; Canalis and Raisz, 1979; Raisz *et al.*, 1976).

Osteogenic cells from embryonic chick calvaria can be grown as an explant of the periosteum (Tenenbaum and Heersche, 1982). Folding the periosteum so the osteogenic side faces inwards onto itself results in osteoblastic differentiation and the formation of osteoid with a concomitant increase in alkaline phosphatase activity; these parameters of phenotypic expression are dependent upon the initial site of the periosteum *in ovo*, eg ecto- or endocranial (McCulloch *et al.*, 1989). The osteoid will mineralise in the presence of

organic phosphate to produce a mineralised matrix surrounded by unmineralised osteoid (Tenenbaum, 1981). Unfolded periostea exhibit osteoblastic differentiation only if they are cultured with the osteogenic surface facing the air, indicating a possible development of a microenvironment due to either apposition or restricted diffusion (Tenenbaum and Heersche, 1986).

b) Cell culture

Bone formation in cultures of isolated osteoblastic cells provides models to answer questions about the regulation and mechanisms of osteogenesis at the cellular level. A very early report of bone formation *in vitro* by isolated osteoblastic cells used cultures established by enzymatic digestion of periosteum-free fetal rat calvaria (Binderman *et al.*, 1974). The cells secreted a collagenous matrix that was progressively mineralised. However, the appearance of mineralisation was slow, the mineral crystals bore no spatial relation to the collagen fibres and the pattern of mineralisation was diffuse and presented no mineralisation front.

A later study used cultures established by migration of osteogenic cells away from bone fragments (Ecarot-Charrier *et al.*, 1983). Following an initial period of outgrowth the cells were replated by scraping and thus at no point did the cells come into contact with exogenous digestive enzymes. The cultures secreted an extracellular collagenous matrix (primarily of type I collagen) which showed mineral deposits in the presence of organic phosphate. The calcified matrix contained embedded cells, each surrounded by a layer of osteoid, resembling the *in vivo* situation. Matrix vesicles were also seen within these cultures and these structures may have acted as nucleators although mineral crystals were also seen in association with collagen fibrils.

Bone cells can, therefore, synthesise an osteoid-like extracellular matrix that can support physiological mineralisation, despite the initial disappointing results of Binderman and colleagues (1974). Bone cell populations released by collagenase digestion of fetal calf calvaria can secrete and mineralise an extracellular matrix *in vitro* (Whitson *et al.*, 1984). A later study showed that enzymatically released cells can form bone *in vitro* following

addition of organic phosphate to the culture medium (Nefussi *et al.*, 1985). Periosteum-free fetal rat calvaria were digested using collagenase. The cells released were plated in medium supplemented with both ascorbic acid and ß-glycerophosphate. The cultures were characterised by the early formation (day 4) of multilayered nodules of cells. By day 11 the cultures started to deposit mineral in discrete sites within the nodules. The formation of nodules may be an *in vitro* representation of the developmental appearance of bony nodules during intramembranous osteogenesis.

Structurally, the nodules are formed of cells and extracellular matrix and bear a close correlation to bone *in vivo* (Bhargava *et al.*, 1988). The upper surface of the nodule is covered in a layer of cuboidal osteoblastic cells joined by adherens type junctions (desmosomal). These cells contain abundant cytoplasmic rough endoplasmic reticulum, Golgi complexes and mitochondria. Within the nodule there are osteocytic cells, completely surrounded by matrix. Gap junctions are present between the cell processes which extend into the matrix. The matrix collagen is highly organised and exhibits a closely packed orthogonal arrangement. Mineral crystals are found in association with the collagen fibres, although some matrix vesicles are also present. An unmineralised seam of osteoid lies between the embedded osteocytes and the mineralised matrix.

Thus, bone formed in cell culture exhibits regulated mineralisation associated with organised collagen fibrils and some matrix vesicles, as well as an intricate network of interconnected osteocytic cells within a mineralised matrix overlain by cuboidal osteoblasts.

The number of osteogenic nodules can be influenced by various factors. In the absence of ascorbic acid no nodules form, and in the absence of ß-glycerophosphate no nodules are mineralised (Bellows *et al.*, 1986). Addition of dexamethasone stimulates nodule formation, showing an effect of glucocorticoids upon skeletal tissue. Over long-term culture, EGF causes a highly significant decrease in nodule formation by a prostaglandin-independent mechanism. However, short-term exposure to EGF caused a highly significant increase in nodule formation, possibly due to a mitogenic action on precursor cells.

Undifferentiated chick limb mesenchymal cells can form bone, as well as other connective tissues, following culture *in vitro* (Osdoby and Caplan, 1979). The phenotypic expression by the mesenchymal cells is dependent upon the initial cell density at plating (Osdoby and Caplan, 1980). Chondrogenesis occurs in high density cultures, whereas osteogenesis and mineral deposition occurs at lower densities.

The clonal osteoblastic cell line, MC3T3-E1, is capable of secreting an extracellular matrix that can mineralise *in vitro* (Sudo *et al.*, 1983). By day 18, the banding pattern of the extracellular collagen fibres became apparent and small nodules of osteoblastic cells began to develop by day 21. Matrix vesicles are found at day 24 and these provide nucleation sites for crystal formation. By day 30 mineral crystal starts to align along the collagen fibrils, and osteocytes within the nodules became enveloped in the mineralised matrix. X-ray emmission analysis shows the mineral to be composed of calcium and phosphorous. Addition of ß-glycerophosphate causes a marked increase in the rate of mineral deposition (Kodama *et al.*, 1986).

MC3T3-E1 cells can also form a mineralised tissue following growth and differentiation within a three-dimensional type I collagen gel matrix (Sudo *et al.*, 1986). Crystal-containing matrix vesicles appears at day 15 with subsequent mineralisation of the matrix. Mineralisation is advanced by the addition of ß-glycerophosphate.

c) Marrow culture

The osteogenic properties of marrow stromal cells and the possibility of an osteogenic stem cell are discussed in a recent review by Beresford (1989).

As has been seen, transplantation of marrow results in osteogenesis at the new site followed by invasion of host haemopoietic cells and the formation of a bone organ (Friedenstein, 1976). Marrow stromal cells in diffusion chambers *in vivo* can give rise to both osteoblastic and chondroblastic differentiation and the formation of mineralised bone tissue containing collagen and matrix vesicles (Ashton *et al.*, 1980). Histologically, the tissue resembles primary or woven bone with a disorganised orientation of the collagen. The pattern of formation is similar to that of endochondral osteogenesis during normal

development (Mardon *et al.*, 1987). Measurement of alkaline phosphatase activity and the content of calcium and phosphorous provides a method of quantifying osteogenesis (Bab *et al.*, 1984). Inclusion of demineralised bone matrix stimulates osteogenesis either by the action of bone morphogenetic protein upon a possible IOPC fraction of marrow or by the action of other matrix components upon osteoblastic proliferation and synthetic ability (Green *et al.*, 1986).

Marrow stromal cells maintain their osteogenic potential in diffusion chambers *in vivo* following a period of *in vitro* growth (Friedenstein *et al.*, 1987). Fibroblast colonies can be established *in vitro* from bone marrow suspensions. Transplantation of cells from either one or several colonies results in the formation of bone and cartilage in diffusion chambers *in vivo*. This shows that within the original population of fibroblast colony-forming cells there exists an osteogenic potential indicating the presence of osteogenic stem cells. These stem cells are not distributed evenly throughout the marrow cavity. The efficiency of the marrow cells to form fibroblastic colonies *in vitro* increases through the sequence core and intermediate marrow towards the endosteal surface, where it is highest (Ashton *et al.*, 1984). Correspondingly, osteogenic marrow stroma cells are more abundant towards the bone surface.

Organ cultures of adult marrow can form bone *in vitro* (Luriya *et al.*, 1986). Marrow was cultured as explants on rafts of Millipore filters at the gas/liquid interface. The medium was supplemented with both ascorbic acid initially and with ß-glycerophosphate at day 8. Within the explant, condensations of cells formed, which developed into thin trabeculae covered in a palisade of osteoblasts. In the presence of organic phosphate the trabeculae mineralised after day 16. The final bony structure consisted of layers of cells surrounded by the mineralised matrix. Ultrastructurally, the matrix consisted of well-banded collagen arranged orthogonally amongst which were seen matrix vesicles (Luria *et al.*, 1987). Bone marrow cultures as intact plugs covered by tissue culture medium in Petri dishes can form bone in the presence of ascorbic acid and ß-glycerophosphate (Schoeters *et al.*, 1988). Osteogenesis also occurs following culture of marrow cell

suspensions applied to gelatin sponges, permitting a comparison between the initial number of marrow cells and the quantification of osteogenesis (Luria *et al.*, 1988).

Maintenance of the spatial arrangement of marrow cells is not necessary for the expression of osteogenic potential. A single cell suspension produced by density gradient centrifugation of marrow cells can form osteogenic colonies following culture on adherent cells derived from marrow (Tibone and Bernard, 1982). The osteogenic structures consist of a mineralised matrix of collagen containing matrix vesicles, within which are embedded osteocytes. The number of osteogenic colonies formed bears a linear relationship to the number of cells plated, indicating the possible clonal origin of the structures from CFU-O (colony forming units - osteogenic), probably a DOPC. The term CFU-O has also been used to describe the cells within cultures derived from calvarial cells that are capable of initiating bone nodule formation (Bellows and Aubin, 1989).

In the absence of an adherent layer, marrow cells form fibroblastic colonies, a few of which express alkaline phosphatase activity (Owen *et al.*, 1987). This indicates a heterogeneity amongst the colony forming population and also shows that the majority are very undifferentiated cells close to, if not members of, the stem cell compartment. In this system, the expression of alkaline phosphatase can be affected by exogenous factors; it is decreased by EGF and increased by hydrocortisone. This shows the prevention of differentiation by EGF and the activation or induction of osteogenic differentiation by hydrocortisone in otherwise undifferentiated colonies.

Subculture of fibroblastic colonies derived from marrow stroma results in the formation of confluent multilayers capable of supporting osteogenic differentiation and the formation of bony nodules (Maniatopoulos *et al.*, 1988). The nodules show intense alkaline phosphatase activity and consist of a mineralised collagenous matrix containing osteocytes and overlain by osteoid and rows of cuboidal cells. The mineralised nodules form only in the presence of dexamethasone and organic phosphate. There is therefore a component within bone marrow stroma capable of forming bone *in vitro* without the maintenance of the cellular microenvironment.

In summary, osteogenic cells are found throughout the marrow cavity, but with increasing frequence laterally, and also in association with the bone surface. There are many methods to study osteogenesis experimentally and these offer a basis for an understanding of the complex regulation of bone formation.

MATERIALS AND METHODS

LIST OF CONTENTS

A. ANIMALS 41

B. MATERIALS 41

C. BUFFER PREPARATION METHODS
1. Buffered saline 44
i) Ca/Mg free phosphate buffered saline (CMF-PBS) 44
ii) Phosphate buffered saline (PBS) 44
iii) Tyrode's solution 44
iv) Kreb's solution 45

D. CELL PREPARATION
1. Method I 45
i) Dissecting medium 45
ii) Growth medium 45
iii) Enzyme solutions 45
iv) Dissection 46
v) Digestion 46
2. Method II 46
i) Dissecting medium 46
ii) Growth medium 46
iii) Enzyme solutions 47
iv) Dissection 47
v) Digestion 47
3. Method III 48
i) Dissecting medium 48
ii) Growth medium 48
iii) Enzyme solutions 48
iv) Digestion 49
4. Balb/c 3T3 Cell Line 49
i) Derivation 49
ii) Growth medium 49
iii) Handling 50

E. EXPERIMENTAL PROCEDURE
1. Viability 50
2. Adenosine 3':5'-Cyclic Monophosphate Assay 50
i) Assay procedure 50
ii) Calculation of results 51
3. Histochemical Alkaline Phosphatase 52
i) Staining method 52
ii) Primary cells 52
iii) Cultured cells (1 to 5 days) 52
iv) Cultured cells (1 to 14 days) 53
4. Quantitative Alkaline Phosphatase Assay 54
i) Assay calibration 54
ii) Assay procedure 55
iii) Effect of ascorbic acid 55
iv) Effect of organic phosphate 56
v) Effect of hormones 56
a) Insulin 56
b) Dexamethasone 56
vi) Effect of growth factors 56
a) Epidermal growth factor 56
b) Platelet derived growth factor 56

5. Microscopy 57
i) Scanning EM 57
ii) Transmission EM 58
iii) Histology 59
6. Mineralisation *in vitro* 59
i) Experimental procedure 59
ii) Effect of hormones 60
a) Insulin 60
b) Dexamethasone 60
iii) Effect of growth factors 60
a) Epidermal growth factor 60
b) Platelet-derived growth factor 60
7. Quantitative calcium assay 60
i) Assay calibration 60
ii) Assay procedure 61
iii) Effect of organic phosphate 61
iv) Effect of seeding density 61
8. Granulocyte/macrophage colony forming cell assay 62
9. Balb/c 3T3 cell line 62
i) Culture method 62
ii) Preparation of conditioned medium 63
iii) Alkaline phosphatase assay 64
iv) Total protein determination 64
v) Quantitative calcium assay 64

A. ANIMALS

Neonatal CD-1 mice of either sex, unless stated otherwise, housed under standard animal house conditions.

B. MATERIALS

Adenosine 3':5'-cyclic monophosphate, sodium salt (cAMP) - Sigma A-6885

Agar - Bacto-agar "Difco" certified 0140-02

Alkaline phosphatase - from bovine intestinal mucosa Sigma P-7640

Aquacide III - flake polyethylene glycol, Calbiochem 17852

Araldite - Emscope

Ascorbic acid - Sigma A-0278

Aquamount - BDH 'Gurr' 36086

Benzyl penicillin, sodium - Glaxo

Bovine serum albumin - Sigma A-7906

Calcitonin (thyrocalcitonin from bovine thyroid glands) - Sigma T-8135

Calcium chloride ($CaCl_2.2H_2O$) - BDH 'AnalaR' 10071

Calcium/phosphorous combined standards - Sigma 360-11

Catalase - Sigma C-40

Charcoal 'Norit GSX' low in chlorine - BDH 33204

Chloral hydrate - BDH 27668

Citric acid - BDH 'AnalaR' 10081

Collagenase - Worthington's bacterial collagenase, class II Cooper Biomedical

Colourimetric calcium assay - Sigma 587-A

Copper sulphate ($CuSO_4.5H_2O$) - BDH 10091

Dexamethasone (9α-fluoro-16α-methylprednisone) - Sigma D-1756

Dialysis tubing - Medicell International Ltd

Diethanolamine - BDH 'AnalaR' 10393

Disodium hydrogen phosphate (Na_2HPO_4) - BDH 'AnalaR' 10249

Dispase - Protease, neutral grade II, Boehringer Mannheim 165 859

Dithiothreitol - Sigma D-0632

Dulbecco's modification of Eagle's medium (Dulbecco's MEM) - Flow

Eagle's minimum essential medium, Earle's salts (Eagle's MEM) - Flow

Epidermal growth factor - Sigma E-4127

Ethylenediaminetetra-acetic acid, disodium salt - BDH 'AnalaR' 10093

Fast Blue RR salt - C.I. 37155

Fetal calf serum -Gibco

Folin Ciocalteu's reagent - BDH 19058

Formaldehyde - BDH 'AnalaR' 10113

Forskolin, dissolved in ethanol - Sigma F-6886

L-Glutamine - Flow

Glutaraldehyde - 25% solution, BDH 36080

Glycerol - Sigma G-7757

ß-Glycerophosphate - Sigma G-6251

Glycine - BDH 'AnalaR' 10119

Haematoxylin - C.I. 75290

Horse serum - Flow

Insulin - Sigma I-5500

3-Isobutyl-1-methyl-xanthine - Sigma I-5879

Magnesium chloride ($MgCl_2.6H_2O$) - BDH 'AnalaR'10149

Multiwell plates - Linbro, 24 well plates

α-Naphthyl-acid-phosphate, monosodium salt - Sigma N-7000

Neutral red - BDH C.I. 50040

p-nitrophenyl phosphate - Sigma 104

Osmium ampoule - Biorad (Emscope)

Parathyroid hormone - National Institute of Biological Standards and Control, reagent 761572

Perchloric acid - BDH 'AnalaR' 10175

Platelet derived growth factor - Sigma P-8147

Potassium alum ($AlK(SO_4).12H_2O$) - BDH 27085

Potassium chloride (KCl) - BDH 'AnalaR' 10198

Potassium dihydrogen phosphate (KH_2PO_4) - BDH 'AnalaR' 10203

Propylene oxide - BDH 28290

Silver nitrate ($AgNO_3$) - Sigma S 6506

Sodium cacodylate - BDH 30118

Sodium carbonate (Na_2CO_3) - BDH 1024

Sodium chloride (NaCl) - BDH 'AnalaR' 10241

Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) - BDH 'AnalaR' 120245

Sodium hydrogen carbonate ($NaHCO_3$) - BDH 10247

Sodium hydroxide (NaOH) - BDH

Sodium iodate ($NaIO_3.H_2O$) - BDH 'GPR' 30171

Sodium thiosulphate ($Na_2S_2O_3.5H_2O$) - BDH 30235

Streptomycin sulphate - Evans Medical Ltd

Sucrose - Fisons

Tartaric acid D (+) - BDH 10277

Theophylline (1,3-dimethylxanthine, phosphodiesterase inhibitor) - Sigma T-1633

Tris(hydroxymethyl) methylamine - BDH 'AnalaR' 10315

Trypan blue stain - Sigma T-9520

Trypsin - Difco

C. BUFFER PREPARATION METHODS

1. Buffered saline

i) Ca/Mg free phosphate buffered saline (CMF-PBS)

KH_2PO_4	0.4g/litre
Na_2HPO_4	5.0g/litre
NaCl	20.0g/litre
KCl	0.5g/litre

Dissolved in distilled water, pH adjusted to 7.4.

ii) Phosphate buffered saline (PBS)

KH_2PO_4	200mg/litre
Na_2HPO_4	1150mg/litre
NaCl	8000mg/litre
KCl	200mg/litre
$MgCl_2.6H_2O$	100mg/litre
$CaCl_2.2H_2O$	132.5mg/litre

Dissolved in distilled water, pH adjusted to 7.4.

iii) Tyrode's solution

KCl	200mg/litre
NaCl	8,000mg/litre
$NaHCO_3$	1000mg/litre
$NaH_2PO_4.2H_2O$	5.0mg/litre

Dissolved in distilled water, pH adjusted to 7.5.

iv) Kreb's solution

NaCl	8.0g/litre
KCl	0.402g/litre
CaCl	0.412g/litre
$MgSO_4.7H_2O$	0.296g/litre
$NaH_2.PO_4$	0.047g/litre
$KH_2.PO_4$	0.041g/litre
1N HCl	12mls
Glucose	2.0g/litre

The pH was adjusted to 7.4 at 37°C using 14mM TRIS solution.

D. CELL PREPARATION

1. Method I

i) Dissecting medium

The dissecting medium was Eagle's MEM supplemented with 0.5% BSA and 200U/ml catalase.

ii) Growth medium

The growth medium was Eagle's MEM supplemented with 10% FCS, 50IU/ml benzyl penicillin, 50μg/ml streptomycin sulphate, 2mM L-glutamine, amino acids, vitamins, 10mM ß-glycerophosphate and 50μg/ml ascorbic acid.

iii) Enzyme solutions

Two enzyme solutions were used. Collagenase (class II) at approximately 400U/ml dissolved in CMF-PBS containing 4mM EDTA. Trypsin made up to 0.1% in 2mM EDTA dissolved in CMF-PBS.

Both enzyme solutions were sterilised by filtration through a 0.22μm Millipore filter.

iv) Dissection

Ten neonatal mice were stunned by chilling in crushed ice and then killed by decapitation. Using flame-sterilised instruments in a laminar flow hood the scalp was completely removed to expose the calvaria. The bones were crudely dissected out and transferred to a sterile glass petri dish containing dissecting medium. The bones were then removed from this and transferred into sterile PBS in a petri dish under a dissecting microscope. All the sutures were carefully cut away and placed in 1ml of trypsin solution. The periosteum was then peeled off the bones and transferred to another 1ml of trypsin. The cleaned calvaria were then placed in a separate sterile glass petri dish containing dissecting medium.

v) Digestion

The sutures and periostea were incubated in a small sterile tube with continuous agitation for 30 minutes at 37°C at which time the supernatant was aspirated. The calvaria were incubated in a similar manner for four periods of 30 minutes, each in 1ml of collagenase. At the end of each collagenase digestion the supernatant was aspirated and replaced with a similar volume of fresh enzyme solution.

2. Method II

i) Dissecting medium

The dissecting medium was a modified form of BGJ medium containing 220mg/100mls sodium hydrogen carbonate (Reynolds, 1976). The medium contained 50IU/ml benzyl penicillin, 50μg/ml streptomycin sulphate and 2mM L-glutamine.

ii) Growth medium

The growth medium was Dulbecco's MEM supplemented with 10% FCS, 50IU/ml benzyl penicillin, 50μg/ml streptomycin sulphate and 2mM L-glutamine.

iii) Enzyme solutions

Three enzyme solutions were used. Collagenase at approximately 400U/ml dissolved in Tyrode's solution. Dispase dissolved in Tyrode's solution at 2mg/ml. Trypsin made up to 0.1% in Tyrode's solution.

iv) Dissection

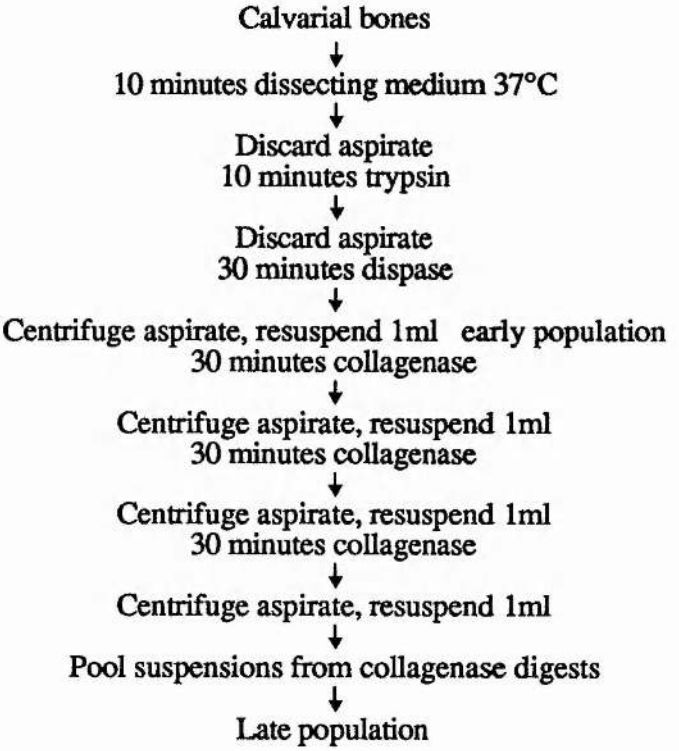
Neonatal mice were killed by decapitation in lots of 3 to 5. The severed heads were then sterilised by brief immersion in 70% alcohol and the following procedures were then done under sterile conditions. The instruments were autoclaved before use and during use the sterility was maintained by dipping in 70% alcohol followed by flaming.

The scalp was peeled forwards to expose the calvarial bones. Using a pair of scissors the bones were crudely removed and transferred to a sterile glass petri dish containing dissecting medium. Under a dissecting microscope the sutures around the temporal and parietal bones were removed, including the lamboid suture and the interparietal bone, leaving the coronal and saggital sutures in place. The saggital suture was then removed by cutting very closely either side of it with a scalpel blade, taking care not to harm the periosteum. The calvarial halves were then transferred to another sterile glass petri dish containing dissecting medium.

v) Digestion

The bones were chopped up using a pair of scissors and transferred to a small sterile glass bottle containing a magnetic flea and 1ml/10 mice of dissecting medium. The magnetic stirrer was situated in a room maintained at 37°C. The following digestion procedure was used: (1) 10 minutes dissecting medium, (2) 10 minutes trypsin, (3) 30 minutes dispase, (4) to (6) 3 x 30 minutes collagenase. Each time the supernatant was aspirated and the same volume of fresh digestion mixture was added to the calvaria. The first two aspirates were usually discarded. The dispase aspirate was centrifuged for 5 minutes at 300g and the pellet resuspended in 1ml of growth medium; this cell suspension contained what was designated the early population of cells. The aspirates from the three

collagenase digests were treated in similar manner except that they were pooled following centrifugation and resuspension in 1ml.



3. Method III

i) Dissecting medium

The dissecting medium was prepared as method II.

ii) Growth medium

The growth medium was prepared as method II.

iii) Enzyme solutions

The enzyme solutions used were dispase and collagenase (200U/ml), prepared as method II.

iv) Digestion

The calvarial halves were chopped once using a scalpel under the dissecting microscope along a line just caudal to the coronal suture. The fragments were then transferred to a small sterile plastic vial containing a magnetic flea and 1ml/10 mice of dissecting medium. The bones were then incubated at 37°C for 10 minutes. The magnetic stirrer used was capable of maintaining a much slower rate of revolution of the magnetic flea than that used in method II. The dissecting medium was discarded and replaced with an equivalent volume of dispase for 30 minutes. Following this there were three 30 minute digestions using collagenase. The aspirates from these digests were pooled to give one population of bone cells.

Calvarial bones
↓
10 minutes dissection medium, 37°C
↓
Discard aspirate, 30 minutes dispase
↓
Discard aspirate, 30 minutes collagenase
↓
Centrifuge aspirate, resuspend in 4mls growth medium
30 minutes collagenase
↓
Centrifuge aspirate, resuspend in 1ml
30 minutes collagenase
↓
Centrifuge aspirate, resuspend with
pooled suspensions (total 5mls)
↓
Single bone cell suspension

4. Balb/c 3T3 Cell Line

i) Derivation

The cell line, 3T3 clone A31, was derived from Balb/c mouse embryos and was purchased from Flow.

ii) Growth medium

The growth medium was prepared as method II.

iii) Handling

The cultures were initially passaged at day 3 following seeding at 2.0 x 10^5 per 9cm Nunclon petri dish. The medium was aspirated and the cell layer was washed with 3.5mls of CMF-PBS. The plate was then incubated at 37°C with 1ml of trypsin solution made up to 0.1% in CMF-PBS containing 1mM EDTA. After 5 minutes 3mls of growth medium were added to inhibit the enzyme reaction and the resulting solution was sucked up and down using a sterile pipette before being transferred to a centrifuge tube. The cell suspension was then centrifuged for 3 minutes at 380g. The pellet was resuspended in growth medium and plated.

E. EXPERIMENTAL PROCEDURE

1. Viability

A viability test was performed on each separate aspirate produced using method II. Sterile 0.4% trypan blue stain was diluted to 0.02% with sterile PBS. This was then mixed 1:1 with the cell suspension and incubated at room temperature. Viability was assessed by counting approximately 100 cells under a light microscope and determining the percentage that were capable of excluding the dye. Dye exclusion was assumed to correspond to viability.

2. Adenosine 3':5'-Cyclic Monophosphate Assay

i) Assay procedure

The assay method used was based on that published by Brown and colleagues (1971). A cell suspension of bone cells was prepared using method III and was seeded into a multiwell plate at 2.0 x 10^5 cells/well in growth medium supplemented with 50μg/ml ascorbic acid. The assay was performed at day 7 on fully confluent cultures. The media were aspirated and the cell layers were washed with Kreb's salt solution. The Kreb's solution was aspirated and 500μl of incubation medium was added to each well; (1) 1mM IBMX dissolved in Kreb's containing 1% BSA, (2) 10μM forskolin dissolved in Kreb's

containing 1mM IBMX and 1% BSA, (3) 1mU/ml calcitonin dissolved in Kreb's containing 1mM IBMX and 1% BSA, and (4) 1U/ml PTH dissolved in Kreb's solution containing 1mM IBMX and 1% BSA. The cultures were incubated at 37°C for 15 minutes. The reaction was halted by first washing the cell layers with ice-cold Kreb's and then incubation with 150μl of ice-cold 0.2N HCl per well for 15 minutes. The acid was then neutralised with 105μl of 0.5M TRIS to give a final pH of 7.5 and the supernatant and cell debris were transferred to small numbered tubes and then centrifuged at approximately 940g for 15 minutes at 4°C. Aliquots (50μl) of the supernatant were incubated at 4°C for 1.5 to 24 hours with 50μl of tritiated cAMP (5μCi/10mls) and 200μl of binding protein solution (binding protein dissolved in 50mM TRIS solution containing 5mM $MgCl_2$ and approximately 8 to 10mM theophylline and a trace of dithiothreitol). Standards containing known amounts of cold cAMP (0 to 40pmoles and 1mmole) were also incubated and subsequently treated in a similar manner.

At the end of the incubation period 500μl aliquots of a suspension of 2% charcoal in distilled water containing 0.2% BSA were added to each assay tube and then centrifuged at approximately 940g for 15 minutes at 4°C. The supernatant was mixed with scintillant and the radioactivity was counted at 5 minutes per vial on an LKB Wallac 1214 Rackbeta liquid scintillation counter.

ii) Calculation of results

At 1mM of cold cAMP no [^{3}H]-cAMP binds to the binding protein, this is therefore the blank value. At 0mM of cold cAMP the maximum amount of [^{3}H]-cAMP binds; subtracting the blank value from this gives the maximum possible binding of radioactivity, termed C_o. Subtracting the blank value from the counts per minute for the standards and samples gives a value, C_x.

3. Histochemical Alkaline Phosphatase

i) Staining method

The slides were air dried and then fixed in acetone at 4°C for 7 minutes. Following fixation the slides were washed twice with tap water and then incubated for 20 minutes at room temperature in substrate solution: 10mg of substrate (α-naphthyl acid phosphate), 10mg catalyst ($MgCl_2.6H_2O$) and 10mg diazotised coupler (Fast Blue RR salt) were dissolved in 10mls of 0.05M TRIS buffer at pH 10.0. The slides were then washed with tap water for 1 minute before incubation with Mayer's Haemalum for 2 minutes. The nuclei were then blued with tap water and the slides were mounted using Aquamount.

The Mayer's Haemalum was prepared by dissolving overnight in 1 litre of distilled water 50g potassium alum, 1g haematoxylin and 0.2g sodium iodate. The following day 1g citric acid and 50g chloral hydrate were added and the solution was boiled for 5 minutes in a fume cupboard. The solution was cooled and filtered before use.

ii) Primary cells

Cell suspensions were prepared following method I to produce six populations; sutures, periostea and four successive populations derived from collagenase digestion of the calvarial bones. The cell populations were further subdivided with respect to the age of the source animals. The neonates were used at ages 0 to 5 days. Following enzymatic digestion the aspirate was centrifuged at 300g for 10 minutes and the pellet resuspended in 100μl of growth medium. A cytospin was performed on the cell suspension and the slides then stained for alkaline phosphatase.

iii) Cultured cells (1 to 5 days)

Cell suspensions were prepared following method II to produce two populations designated early and late. Cell counts were performed on both populations using a Mod-Fuchs Rosenthal haemocytometer and the cell concentration was adjusted to 5.0×10^5 cell/ml. The cell suspensions were then plated out into 30mm dishes (Sterilin) at 1.5mls/plate. Ascorbic acid was not added to the growth medium. The cultures were

incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 to 5 days. The media were changed every two days.

The primary cell suspension was further diluted to 2.0 x 10^5 cells/ml and cytospins were performed on 200μl aliquots of this suspension. These were then stained for alkaline phosphatase and the percentage of positive cells assessed.

After a period in culture of 1 to 5 days the medium was aspirated and the cell layer washed with 2mls of CMF-PBS. This was removed and replaced with 1ml of 0.1% trypsin in CMF-PBS containing 2mM EDTA and the plates were incubated at 37°C for 5 minutes and checked, using a phase contrast microscope, to ensure that the cells were loosened from the plastic surface. Using a plugged pasteur pipette the trypsin solution was sucked up and washed over the surface of the plate several times. All the liquid was then aspirated into a centrifuge tube containing 3mls of growth medium. The cell suspension was centrifuged at 300g for 5 minutes and the pellet resuspended in 1ml of medium. A cell count was performed on part of this suspension and the concentration was adjusted to 2.0 x 10^5 cells/ml. Cytospins were performed on 200μl aliquots and the slides stained for alkaline phosphatase and the percentage of positive cells assessed.

iv) Cultured cells (1 to 14 days)

Cell suspensions were prepared following method II to produce two populations designated early and late. Cell counts were performed on both populations using a Mod-Fuchs Rosenthal haemocytometer and the cell concentration was adjusted to 2.0 x 10^5 cells/ml. The cell suspension was seeded into multiwell plates at 1ml/well. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 to 14 days, the media were changed every two days.

Cytospins were performed on 200μl aliquots of the primary cell suspension and these were then stained for alkaline phosphatase and the percentage of positive cells was assessed.

During the incubation period the growth media were supplemented with;

a) 50μg/ml or 100μg/ml ascorbic acid,

b) 10mM ß-glycerophosphate,

c) 50μg/ml ascorbic acid and 10mM ß-glycerophosphate, or

d) 50μg/ml ascorbic acid, 10mM ß-glycerophosphate and 10^{-7}M dexamethasone.

Control cultures were unsupplemented. Other cultures were incubated for 14 days with various concentrations of ascorbic acid (0, 50, 75, 100 and 150 μg/ml).

After a period in culture of 1, 2, 4, 6, 10 or 14 days the medium was aspirated and the cell layers were washed with 1ml CMF-PBS. This was removed and replaced with 0.5ml of 0.1% trypsin solution in CMF-PBS containing 2mM EDTA and the plates were then incubated at 37°C for 5 minutes. Using a plugged pasteur pipette the trypsin solution was sucked up and washed over the floor of the well several times. If the culture was at day 10 or 14 the cultures were washed with CMF-PBS and initially incubated with 0.5ml collagenase (400U/ml) for 30 minutes. This was aspirated and the remaining cells incubated with 0.1% trypsin.

The cells suspension was centrifuged at 300g for 5 minutes and the pellet resuspended. A cell count was performed and the cell concentration adjusted to 2.0×10^5 cells/ml. Cytospins were performed on 200μl aliquots, the slides stained for alkaline phosphatase, and the percentage of positive cells assessed.

4. Quantitative Alkaline Phosphatase Assay

i) Assay calibration

The assay relies upon the ability of alkaline phosphatase to hydrolyse *p*-nitrophenyl phosphate to produce *p*-nitrophenol and inorganic phosphate. At alkaline pH the *p*-nitrophenol is strongly absorbant at a wavelength of 410nm. The method used is based on that of Lowry (1955).

The purchased alkaline phosphatase was dissolved in 1M diethanolamine buffer at 0.1mg/ml and 0.2mg/ml. The phosphatase substrate *p*-nitrophenyl phosphate was dissolved in 1M diethanolamine to a concentration of 15mM.

Ten assay tubes were set up containing 0 to 100μg of alkaline phosphatase. To these were added 200μl aliquots of *p*-nitrophenyl phosphate. Standards of 0 to 0.40 μmole *p*-nitrophenol were treated in a similar manner. The tubes were incubated for 30 minutes at 37°C in a shaking water bath. The reaction was halted by addition of 3.6mls of 0.1M NaOH and the absorbance read at 410nm on a Philips spectrophotometer (PU 8620 UV/VIS/NIR).

ii) Assay procedure

Single cell suspensions were prepared using method III and seeded into multiwell plates and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Following an incubation period of up to 14 days the cultured cells were dissociated using enzymatic means. The media were aspirated and the cultures washed with 1ml CMF-PBS. The plates were then incubated for 30 minutes at 37°C with 1ml collagenase (200U/ml) in each well. The cell suspension was then sucked up and down using a pasteur pipette and transferred to a conical tube. The cell suspension was either used in this state or centrifuged for 5 minutes at 300g and then resuspended in 1ml of distilled water. The cell walls were then broken down by sonification in three bursts of ten seconds each with cooling in between. The sonicate was then incubated as 200 to 400μl aliquots with 200μl of 15mM *p*-nitrophenyl phosphate in 1M diethanolamine for 30 minutes in a shaking water bath at 37°C. Standards of 0.0 to 0.4 μmoles were treated in a similar manner. The reaction was halted by the addition of 3.6mls of 0.1M NaOH and the absorbance read at 410nm.

iii) Effect of ascorbic acid

Cultures were supplemented with 5, 10, 25, 50, 75 and 100μg/ml ascorbic acid and incubated for 7 days or 5, 50 and 75μg/ml for 14 days. Another set of cultures were

supplemented with 50μg/ml ascorbic acid and the alkaline phosphatase content was assayed at day 3, 5, 7, 9, 11 and 13.

iv) Effect of organic phosphate

Cultures were supplemented with 50μg/ml ascorbic acid and 0, 1, 5 and 10 mM ß-glycerophosphate. The alkaline phosphatase content was assayed at day 14.

v) Effect of hormones

a) Insulin

Cultures were established and supplemented with 0, 50, 500 and 5000 ng/ml insulin with and without 50μg/ml ascorbic acid. The alkaline phosphatase content was assayed at day 14.

b) Dexamethasone

Cultures were set up and supplemented with 10^{-7}M dexamethasone with and without 50μg/ml ascorbic acid. Alkaline phosphatase content was assayed at day 14.

vi) Effect of growth factors

a) Epidermal growth factor

Cultures were set up and supplemented with 0, 5, 10 and 20 ng/ml EGF and 50μg/ml ascorbic acid and incubated for 7 days. Other cultures were supplemented with 0, 1, 5, 10 and 20 ng/ml EGF and 50μg/ml ascorbic acid and incubated for 14 days. At the end of the incubation period the cultures were assayed for alkaline phosphatase.

b) Platelet derived growth factor

Cultures were set up and supplemented with 2ng/ml PDGF with and without 50μg/ml ascorbic acid. The alkaline phosphatase content was assayed at day 14.

5. Microscopy

i) Scanning EM

A cell suspension prepared following method III was seeded into multiwell plates at 2.0 x 10^5 cells/well in medium supplemented with 50μg/ml ascorbic acid. The cultures were further supplemented with 10mM ß-glycerophosphate at day 9.

At day 14 the media were aspirated and the cultures washed with a phosphate and calcium free buffer (TRIS 2.42g and 29.24g NaCl in 1000mls distilled water at pH 7.5). This was replaced with an aldehyde fix solution for 30 minutes at 4°C (sucrose 0.6g and 10mls 25% glutaraldehyde solution plus 100mls buffer). The plates were then dehydrated with alcohol (50%, 70%, 80% and 90%, ten minutes each) and then taken through three changes of twenty minutes each of absolute alcohol.

The samples were then critical point dried using a Samdri 780 (Emscope). Critical point drying first replaces the absolute alcohol in the sample with liquid carbon dioxide. This is then taken to the critical point of carbon dioxide, consequently causing the conversion from liquid to gas. Evaporation of liquid carbon dioxide is an exothermic process and would therefore cause heat damage in the sample.

Two different types of coating were used; i) gold, and ii) carbon. Using an SC 500 (Emscope) samples were coated with gold to a depth of 10nm and then subjected to a voltage of 15kV in a JEOL JSM-35CF scanning electron microscope. Images produced using X-ray emission of the Ka of calcium were overlaid onto photographs of the same area to produce speckle pictures representing the calcium distribution. It was not possible to produce a plot of the elemental distribution using this method as the phosphorous peak was too close to that produced by the gold coating. Further samples were therefore coated in carbon to a depth of 10 to 15nm using a 'Speedivac' coating unit 12E6/1688 (Edwards High Vacuum Ltd). These samples were exposed for 130 seconds to a voltage of 15kV and a plot was produced on a Link System XY recorder 26000 A4 via a Link System model 290 with a Kevex series #3000 cryogenic subsystem.

ii) Transmission EM

A cell suspension prepared following method III was seeded at 2.0 x 10^5 cells/well into multiwell plates. The media were supplemented with 50μg/ml ascorbic acid at seeding and with 10mM ß-glycerophosphate at day 9.

At days 7, 10 and 14 the cultures were washed once with cacodylate buffer (0.2M sodium cacodylate, pH 7.3) and fixed for 30 minutes with 2.5% glutaraldehyde solution in cacodylate buffer. The samples were then rinsed twice with buffer and post-fixed for 30 minutes with 1% osmium solution (5mls cacodylate buffer plus the content of an osmium ampoule dissolved in 5mls of distilled water). The samples were again rinsed twice with cacodylate buffer and then dehydrated with alcohol (50%, 70%, 80% and 90%, ten minutes each) and finally taken through three changes of twenty minutes each of absolute alcohol. In preparation for the resin stage, the samples had three changes of twenty minutes each of propylene oxide. This was followed by one hour of two parts propylene oxide/one part resin (27 parts Araldite, 23 parts hardener, 1 part accelerator) and then one hour 2 parts resin/1 part propylene oxide. The samples were then placed in pure resin in foil-coated dishes and hardened at 60°C for 48 hours.

2.5% glutaraldehyde fixation
↓
1% osmium post-fixation
↓
Alcohol dehydration
↓
Propylene oxide
↓
Propylene oxide/resin (2:1)
↓
Propylene oxide/resin (1:2)
↓
Embedded in pure resin

Using a Reichart microtome and a fine glass knife sections 50 to 60nm thick were cut from the samples. These were mounted onto copper grids and stained with Reynold's lead citrate and saturated uranyl acetate in 50% alcohol. The sections were stained first

with lead citrate for 3 minutes, washed with distilled water and then stained with the uranyl acetate for 3 minutes. The grids were again washed with distilled water and then dried with filter paper.

The sections were then visualised using a Philips 301 transmission electron microscope.

iii) Histology

The localisation of insoluble calcium within osteogenic cultures was demonstrated using the von Kossa method. The media were aspirated, the plates rinsed with PBS and the cultures then fixed with 10% formaldehyde in absolute alcohol at 4°C for 30 minutes. The plates were then stained for calcium using 1% aqueous silver nitrate for 15 minutes while exposed to a bright light. Unreduced ionised silver was removed using 5% aqueous sodium thiosulphate for 2 minutes. Counter-staining was with 0.5% aqueous neutral red for 1 minute. The samples were mounted using Aquamount mountant.

Early population cells (prepared following method II) were seeded into 30mm tissue culture dishes (Sterilin). At day 4 the cultures were supplemented with 50μg/ml ascorbic acid and 10mM ß-glycerophosphate. At day 28 the cultures were further supplemented with 10^{-7}M dexamethasone and were fixed at day 42.

6. Mineralisation *in vitro*

i) Experimental procedure

Single cell suspensions were prepared using method III and seeded into multiwell plates and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. ß-glycerophosphate (10mM) was added to the medium at day 9. After a total period in culture of 14 days, the samples were fixed and stained for insoluble calcium using the von Kossa method.

The extent of mineralisation was semi-quantified using a Chalkley grid graticule inserted into a x8 eye-piece of an Olympus inverted microscope. Using a x10 objective, the extent of mineralisation was determined by counting the number of points on the grid

overlying calcified areas in a total of 20 fields per sample. Mineralisation is therefore expressed as a percentage of the total area.

ii) Effect of hormones

a) Insulin

Cultures were established and supplemented with 0, 50, 500 and 5000ng/ml insulin, with and without 50μg/ml ascorbic acid.

b) Dexamethasone

Cultures were set up and supplemented with 10^{-7}M dexamethasone, with 50μg/ml ascorbic acid.

iii) Effect of growth factors

a) Epidermal growth factor

Cultures were set up and supplemented with 0, 1, 5, 10 and 20ng/ml EGF and 50μg/ml ascorbic acid.

b) Platelet-derived growth factor

Cultures were set up and supplemented with 0 and 2ng/ml PDGF with 50μg/ml ascorbic acid.

7. Quantitative calcium assay

i) Assay calibration

The assay is based on the ability of calcium to react with o-cresolphthalein complexone resulting in a purple reaction product at alkaline pH. The absorbance is read at 575nm and the colour intensity is proportional to the calcium concentration.

Using purchased calcium/phosphorous combined standards solutions of calcium from 0 to 150μg/ml were made up. Aliquots of these standards were added to 2.5mls of

calcium assay solution in a cuvette. The absorbance of the assay solution at 575nm was read before and after addition of the standard, using distilled water as the blank.

ii) Assay procedure

Single cell suspensions were prepared using method III and seeded into multiwell plates at 2 x 10^5/well and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Following the incubation period of 14 days the cultured cells were dissociated using 1ml/well of collagenase (200U/ml) for 30 minutes at 37°C. The suspension was then transferred to a weighed 10ml centrifuge tube and sonicated in three 10 second bursts. The tubes were then transferred to an LSL Secfroid to freeze dry over night.

The tubes were then reweighed and the sample resuspended in 500μl of 0.4M perchloric acid, vortex mixed, and incubated at 4°C for 2 hours. The samples were then centrifuged at 1800g for 15 minutes and 400μl of the supernatant used for the assay.

Aliquots of 25μl of the sample and 10mg/dL calcium standard were added to 2.5mls of calcium assay solution. The absorbance at 575nm was read before and after addition using distilled water as the blank.

iii) Effect of organic phosphate

Cultures were supplemented with 50μg/ml ascorbic acid and 0, 1, 5 and 10mM ß-glycerophosphate.

iv) Effect of seeding density

Single cell suspensions prepared using method III were seeded into multiwell plates at 1ml/well at cell concentrations of 0.1, 0.5, 1.0, 2.0 and 4.0 x 10^5/ml. The cultures were supplemented with 50μg/ml ascorbic acid at seeding and 10m ß-glycerophosphate at day 8.

8. Granulocyte/macrophage colony forming cell assay

The media consisted of Dulbecco's MEM supplemented with 50IU/ml benzyl penicillin, 50μg/ml streptomycin sulphate, 2mM L-glutamine and 20% horse serum. To prepare a single cell suspension of bone marrow cells, the femur of a six week old male CD-1 mouse was flushed through with medium. The suspension was centrifuged at 300g for 5 minutes and the pellet resuspended in medium and the cell concentration adjusted to 5 x 10^5 to 2 x 10^6/ml. This was then diluted x10 with medium containing 0.3% agar and 1ml aliquot were plated out into 30mm non-tissue culture petri dishes. The cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

Experimental plates were established using conditioned media in place of Dulbecco's MEM. The conditioned media were collected from cultures of osteoblast-like cells prepared using method III seeded at a density of 2.0 x 10^5 cells/ml into multiwell plates in media supplemented with 50μg/ml ascorbic acid. The media were changed every other day, centrifuged to remove most loose cells and were then put to the GM-CFC assay. Before use the conditioned media were supplemented with 20% horse serum. Plates for standardisation were set up with 100μl of WEHI-3 conditioned medium per plate. The WEHI-3 conditioned medium was acquired by taking the supernatant from a mouse myelomonocytic cell line (WEHI-3) grown in liquid culture.

At the end of the incubation period the number and type of colonies was assessed using a low power microscope. The colony morphology corresponded to the cellular composition; macrophages formed loose colonies, granulocytes formed tight colonies and mixed colonies consisted of both cell types (Metcalf *et al.*, 1966).

9. Balb/c 3T3 cell line

i) Culture method

The cells were plated at a cell concentration of 2.0 x 10^5/ml into multiwell plates at 1ml/well. The medium was supplemented with 50μg/ml ascorbic acid. Two days following seeding the cultures were further supplemented with conditioned medium at concentrations of 0, 10, 25, 50, 100 and 500μg total protein/ml. The media were replaced

every other day with fresh media containing both ascorbic acid and conditioned medium. At day 8 onwards the cultures were further supplemented with 10mM ß-glycerophosphate.

The 3T3 cells were cultured for 15 days. The media were aspirated, the cultures washed with CMF-PBS and then incubated for 150 minutes with 1ml/well collagenase (200U/ml). The collagenase was then replaced with trypsin for 5 minutes. The total number of cells dissociated from each well was counted using a Mod-Fuchs Rosenthal haemocytometer. The cell suspensions were sonicated in three 10 second bursts and then split for quantitative analysis of calcium and protein content and of alkaline phosphatase activity.

ii) Preparation of conditioned medium

Conditioned media were collected from osteogenic cultures of cells prepared following method III. The media were pooled and concentrated by dialysis against Aquacide III at 4°C. The dialysis tubing contained pores with an average diameter of 24 Å. The tonicity of the medium was then restored by dialysis against PBS at 4°C.

The total protein content of the concentrated conditioned medium was determined by using a method adapted from Lowry and colleagues (1951). Three solutions were made up: Solution A, 2mls of 10N NaOH and 10mls of 2M Na_2CO_3 were made up to 100mls with distilled water; Solution B, 10mls 1% $CuSO_4.5H_2O$ and 10mls 1.3% tartaric acid were made up to 100mls with distilled water; Solution C, 1:4 dilution of Folin Ciocalteu's reagent with distilled water. In a spectrophotometer cuvette, 1ml solution A and 250μl solution B were mixed. The sample was then added as a 500μl aliquot following a x10 dilution with PBS. Solution C, 250μl, was added last and the cuvettes were then incubated at room temperature for 30 minutes in the dark. Standards of bovine serum albumin (0.2mg/ml to 2.0mg/ml in PBS) were treated in a similar manner. Following the incubation period the absorption was read at 660nm, using a cuvette with no added protein as a blank.

iii) Alkaline phosphatase assay

Up to 350μl of the sonicated sample were assayed for alkaline phosphatase activity.

iv) Total protein determination

The total protein content was assayed on 50μl aliquots of the sonicated sample.

v) Quantitative calcium assay

The sonicate was added to weighed 10ml centrifuge tubes as 1ml aliquots, frozen, and then freeze dried over night. The tubes plus the contents were then reweighed and the samples resuspended in perchloric acid, as before. The calcium content was then determined.

RESULTS

LIST OF CONTENTS

1. Viability 66

2. Adenosine 3':5'-cyclic monophosphate assay 66

3. Histochemical Alkaline Phosphatase 67
   i) Primary cells 67
   ii) Cultured cells (1 to 5 days) 68
   iii) Cultured cells (1 to 14 days) 70

4. Quantitative alkaline phosphatase assay 75
   i) Assay calibration 75
   ii) Effect of ascorbic acid 77
   iii) Effect of organic phosphate 78
   iv) Effect of hormones 80
      a) Insulin 80
      b) Dexamethasone 80
   v) Effect of growth factors 81
      a) Epidermal growth factor 81
      b) Platelet-derived growth factor 81

5. Microscopy 84
   i) Scanning EM 84
   ii) Transmission EM 84
   iii) Histology 85

6. Mineralisation *in vitro* 96
   i) Effect of hormones 96
      a) Insulin 96
      b) Dexamethasone 96
   ii) Effect of growth factors 98
      a) Epidermal growth factor 98
      b) Platelet-derived growth factor 98

7. Quantitative calcium assay 98
   i) Assay calibration 98
   ii) Effect of organic phosphate 98
   iii) Effect of seeding density 100

8. Granulocyte/macrophage colony forming cell assay 102

9. Balb/c 3T3 cell line 104
   i) Conditioned medium 104
   ii) Alkaline phosphatase assay 104
   iii) Protein content 104
   iv) Calcium deposition 104

RESULTS

1. Viability

Cell preparation following method II produced six populations, each corresponding to subsequent steps during the digestion procedure. The percentage viability from the first two populations was very low. In following preparations using this method the first two populations were always discarded. Populations 3 to 6 had percentage viability of ≥95% (Table 3).

Population	Cell Viability	Cell Number
1. Dissecting Medium (10 mins)	8%	0.7
2. Trypsin (10 mins)	12%	1.0
3. Dispase (30 mins)	95%	20.0
4. Collagenase (30 mins)	97%	31.0
5. Collagenase (30 mins)	98%	13.0
6. Collagenase (30 mins)	96%	1.2

Table 3. Viability and number of cells produced during method II for cell preparation.

Cell numbers shown as number x 10^{-5}.

2. Adenosine 3':5'-cyclic monophosphate assay

The concentration of cAMP within the samples can be determined from the regression equation of C_o/C_x against cAMP concentration for the standards. On the basis

that $C_o/C_x = 1$ when [cAMP] is zero, a regression equation was obtained from the data (coefficient of determination = 96.0%);

$$\text{pmoles} = 4.18(C_o/C_x) - 4.18$$

The cAMP concentration of the samples was calculated using this regression equation.

Samples incubated solely with IBMX produced too little cAMP to give an accurate value within the limitations of the assay. Forskolin stimulated cAMP production to such an extent that it was too high for the assay; this did, however, indicate the ability of bone cells to produce cAMP in response to appropriate stimulation. Hormonal stimulation with CT and PTH caused negligible and extensive stimulation respectively (Table 4)

Hormone	[cAMP]
CT	3.1±1.2
PTH	140.5±33.2

Table 4. Stimulation of cAMP production in bone cells by calcitonin and parathyroid hormone.

[cAMP] is expressed as pmoles/10^6 cells. Mean ± standard error of the mean, n = 4.

3. Histochemical Alkaline Phosphatase

i) Primary cells

The expression of alkaline phosphatase by cells associated with bone is characteristic of the osteoblastic phenotype (Martin *et al.*, 1988; Peck and Woods, 1988; Rodan and Rodan, 1984; Sodek and Berkman, 1987). This specificity of enzyme expression allows the use of alkaline phosphatase as an osteoblastic marker.

To assess the proportion of osteoblastic cells, differential counts were performed on the slides of cytospins of cells extracted using method I to give the percentage of the total of cells positive for alkaline phosphatase. No overall difference in alkaline phosphatase

content was observed with respect to the age of the neonatal mice, and the data were therefore pooled (Table 5).

Tissue	Alkaline phosphatase	n
Periosteum	15.3±2.4	17
Sutures	8.3±0.7**	18
Pop^n I	17.7±2.7	18
Pop^n II	34.3±3.8**	16
Pop^n III	4.7±2.3**	17
Pop^n IV	33.7±3.0**	18

Table 5. Percentage primary cells positive for alkaline phosphatase extracted from neonatal mice following method I.

Alkaline phosphatase content shown as the final relative percentage. Mean ± standard error of the mean, ** $p < 0.01$.

The results showed the osteoblast to be more abundant in digests from the calvarial bones than from those of the periostea or the sutures. This indicated the need to dissect away, as carefully as possible, the sutures from the calvaria before the bones were subjected to enzymatic digestion. Due to the close contact between the periostea and the calvarial surfaces, removal of these membranes could easily cause damage to the bone surfaces. Mechanical periosteal removal was therefore not attempted.

ii) Cultured cells (1 to 5 days)

The percentage of alkaline phosphatase positive cells was assessed before seeding of the culture and at the end of the culture period following trypsinisation. Before plating 59.9% ± 1.5 (mean ± standard error of the mean, n = 25) of the early population and 54.0% ± 2.1 (n = 21) of the late population expressed alkaline phosphatase. A Student's t

test to compare these two sets of data showed the populations to be significantly different ($p < 0.05$).

As the original alkaline phosphatase content exhibited a variation, even though small, the final alkaline phosphatase content was expressed as a relative value (final/original) rather than an absolute value.

The cell number of each culture at trypsinisation was also assessed. This gave an indication of the ability of each cell population to adapt to culture conditions.

No overall difference was observed either in alkaline phosphatase content or in cell number recovered with respect to the age of the neonatal mice, and the data were therefore pooled (Tables 6a and 6b).

Days in culture	Alkaline phosphatase	Cell numbers
1	82.8±3.1	2.53±0.18
2	65.2±4.6**	4.08±0.44**
3	31.8±3.0**	8.90±0.92**
4	25.9±2.5**	8.19±0.65**
5	31.5±3.9**	12.73±1.10**

Table 6a. Table showing the alkaline phosphatase content and cell numbers, after a period in culture of cells of the early population prepared following method II.

Alkaline phosphatase content shown as the final relative percentage. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, n =15, * $p < 0.05$, ** $p < 0.01$.

Days in culture	Alkaline phosphatase	Cell numbers
1	56.9±6.1	2.30±0.28
2	46.3±1.5	3.05±0.41
3	34.9±3.0**	6.24±0.90**
4	37.1±4.7*	6.88±0.95**
5	46.9±6.5	8.13±1.10**

Table 6b. Table showing the alkaline phosphatase content and cell numbers, after a period in culture of cells of the late population prepared following method II.

Alkaline phosphatase content shown as the final relative percentage. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, n =15, * $p < 0.05$, ** $p < 0.01$.

On the basis of these data, subsequent cell preparations used neonatal mice less than 5 days old, as judged by their lack of hair eruption. The data also showed the ability of the bone cell preparations to proliferate under culture conditions while maintaining reasonable levels of alkaline phosphatase.

iii) Cultured cells (1 to 14 days)

Before plating, 65.9% ± 2.4 (mean ± standard error of the mean, n = 12) of the early population and 65.5% ± 2.2 (n = 14) of the late population expressed alkaline phosphatase. A Student's t test to compare these two sets of data showed there was no statistically significant difference between them.

Statistical tests using two way analysis of variance showed that overall, following culture, there was no significant difference between the two populations and the data were therefore pooled.

Unsupplemented cultures showed a steady and gradual decline in alkaline phosphatase content over the 14 day culture period. Two way analysis of variance of experimental against control data showed a highly significant ($\alpha < 0.01$) increase in alkaline phosphatase content caused by ascorbic acid (Figure 4). Addition of ß-glycerophosphate also significantly stimulated alkaline phosphatase content, but the effect was dissipated with time in culture (Figure 5). However, in the presence of ascorbic acid, ß-glycerophosphate caused a significant decrease ($\alpha < 0.05$) in alkaline phosphatase levels (Table 7). Addition of dexamethasone to cultures containing ascorbic acid and ß-glycerophosphate caused a significant increase ($\alpha < 0.05$) in alkaline phosphatase.

Experimental *vs* control	α
ß-GP with ascorbic acid and dexamethasone	< 0.01
Ascorbic acid (50μg/ml) *vs* ß-GP with ascorbic acid	< 0.05
ß-GP with ascorbic acid (50μg/ml) *vs* ß-GP with ascorbic acid and dexamethasone	< 0.05

Table 7. Comparison using two way analysis of data of supplemented cultures, in terms of alkaline phosphatase.

Figure 4. Graph showing the effect of ascorbic acid upon alkaline phosphatase content.

Alkaline phosphatase content is shown as the final relative percentage. Time is in days. Mean ± standard error of the mean, n = 8. Two way analysis of variance of experimental (ascorbic acid) against control data shows a highly significant ($\alpha < 0.01$) increase in alkaline phosphatase due to the action of ascorbic acid.

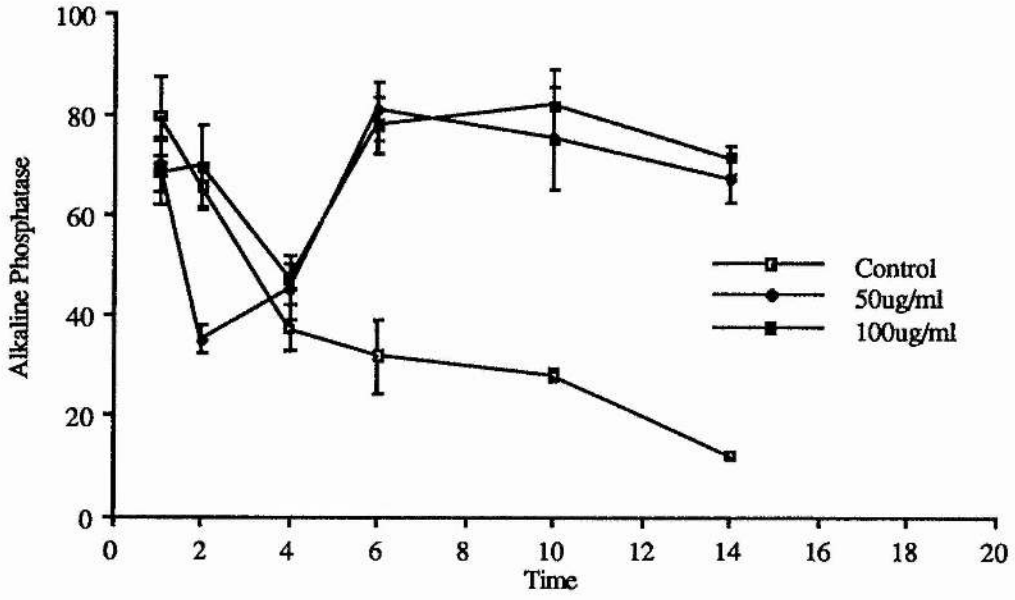
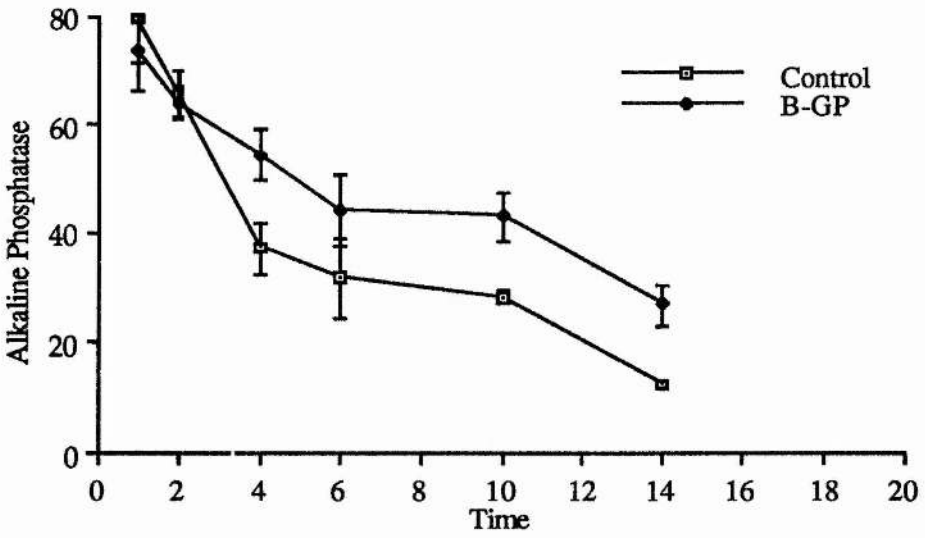


Figure 5. Graph showing the effect of ß—glycerophosphate upon alkaline phosphatase content.

Alkaline phosphatase content is shown as the final relative percentage. Time is in days. Mean ± standard error of the mean, n = 8. Two way analysis of variance of experimental (ß-glycerophosphate) against control data shows a highly significant ($\alpha < 0.01$) increase in alkaline phosphatase due to the action of ß-glycerophosphate.



The addition of 50μg/ml ascorbic acid caused a significant increase ($\alpha < 0.05$) in cell numbers; however, 100μg/ml ascorbic acid and ß-glycerophosphate had no effect (Table 8). Dexamethasone added to cultures containing ascorbic acid and ß-glycerophosphate had no effect on cell number.

Experimental *vs* control	α
Ascorbic acid (50μg/ml)	< 0.05
Ascorbic acid (100μg/ml)	-
ß-glycerophosphate (ß-GP)	-
ß-GP with ascorbic acid (50μg/ml) *vs* ß-GP with ascorbic acid and dexamethasone	-

Table 8. Comparison using two way analysis of variance of supplemented cultures, in terms of final cell number.

After 14 days in culture all concentrations of ascorbic acid produced a highly significant ($p < 0.01$) increase in alkaline phosphatase. At no concentration was there any effect upon cell numbers (Table 9).

Ascorbic acid (μg/ml)	Alkaline Phosphatase	Cell Number
0	12.4±0.8	7.32±1.14
50	67.0±4.7	9.04±0.87
75	73.4±4.9	6.87±1.62
100	71.0±2.9	6.09±1.11
150	63.9±5.9	6.73±0.74

Table 9. Alkaline phosphatase content and cell number following 14 days in culture with various concentrations of ascorbic acid.

Alkaline phosphatase content shown as the final relative percentage. Cell numbers are shown as the final number x 10^{-5}. Mean ± standard error of the mean, n = 8.

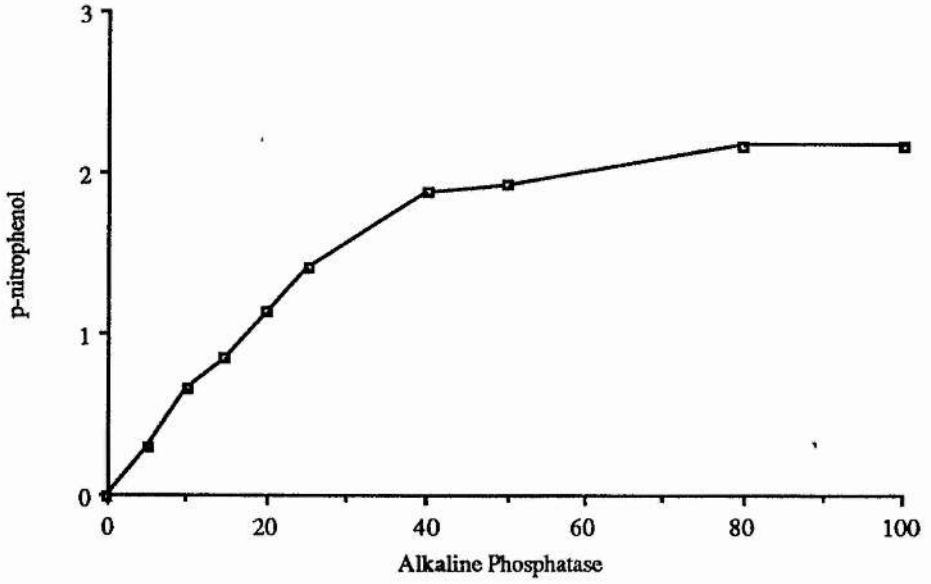
4. Quantitative alkaline phosphatase assay

i) Assay calibration

The correlation of micromoles *p*-nitrophenol released per 30 minutes with micrograms alkaline phosphatase was linear up to a concentration of 25μg alkaline phosphatase per sample (Figure 6). More concentrated samples were therefore diluted before being assayed.

Figure 6. Graph showing the relationship between *p*—nitrophenol release and quantity alkaline phosphatase.

The release of *p*—nitrophenol is shown as micromoles/30 minutes. Alkaline phosphatase is plotted in micrograms.



ii) Effect of ascorbic acid

Analysis using a Student's t test showed that at no concentration of ascorbic acid was there a statistically significant effect upon the total cell number (Tables 10a and 10b). Ascorbic acid did however cause significant increases ($p < 0.05$) in alkaline phosphatase activity at 50, 75 and 100μg/ml following 7 days in culture (Tables 10a and 10b).

A time course study showed a gradual increase in alkaline phosphatase activity up to day 11 followed by a decline (Figure 7).

Ascorbic acid (μg/ml)	Cell number	Alkaline Phosphatase
0	3.50±0.31	3.02±0.48
5	4.27±0.21	4.25±0.40
10	4.45±0.11	4.17±0.55
25	4.92±0.09	3.31±0.15
50	4.41±0.27	4.81±0.35*
75	3.63±0.13	5.72±0.18*
100	2.86±0.28	5.77±0.75*

Table 10a. Effect of varying concentrations of ascorbic acid upon cell numbers and alkaline phosphatase activity following 7 days in culture.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released per 30 minutes per 10^6 cells. Cell number shown as final number x 10^{-5}. Mean ± standard error of the mean, * $p < 0.05$, $n = 4$.

Ascorbic acid (μg/ml)	Cell number	Alkaline Phosphatase
0	7.83±0.57	1.57±0.08
5	8.62±1.10	1.47±0.22
50	8.90±1.00	7.26±1.82
75	8.05±0.83	4.35±1.12

Table 10b. Effect of varying concentrations of ascorbic acid upon cell numbers and alkaline phosphatase activity following 14 days in culture.

Alkaline phosphatase expressed as μmoles *p*-nitrophenol released per 30 minutes per 10^6 cells. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, * $p < 0.05$, $n = 4$.

iii) Effect of organic phosphate

At no concentration of ß-glycerophosphate was there a significant effect upon either cell number or alkaline phosphatase activity (Table 11).

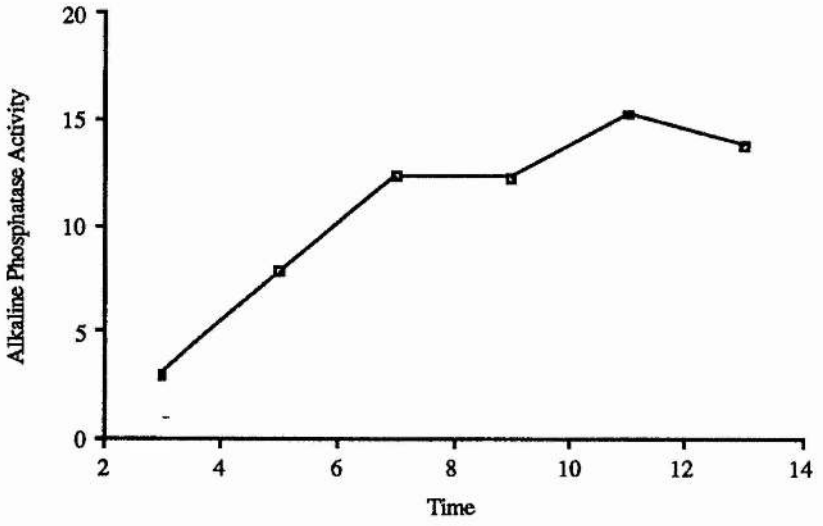
ß-glycerophosphate (mM)	Cell numbers	Alkaline Phosphatase
0	7.25±0.65	8.65±1.09
1	8.43±0.35	11.69±1.49
5	7.03±1.06	9.47±1.72
10	5.98±0.66	7.93±1.05

Table 11. Effect of organic phosphate upon cell numbers and alkaline phosphatase activity.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released per 30 minutes per 10^6 cells. Cell numbers shown as final cell number x 10^{-5}. Mean ± standard error of the mean, $n = 4$.

Figure 7. Graph showing the change in alkaline phosphatase activity with time in culture.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released per 30 minutes per 10^6 cells. Time is shown as days in culture.



iv) Effect of hormones

a) Insulin (with and without ascorbic acid)

At all concentrations of insulin tested, ascorbic acid (50μg/ml) had a highly significant effect ($p < 0.01$) upon both the cell numbers and the activity of alkaline phosphatase. Cultures supplemented with ascorbic acid were completely unresponsive to the addition of insulin. In the absence of ascorbic acid, however, insulin significantly decreased the cell number and increased the alkaline phosphatase activity (Table 12). Stimulation of alkaline phosphatase activity was, however, well below that due to ascorbic acid.

Insulin (ng/ml)	With ascorbic acid Cell number	ALP	Without ascorbic acid Cell Number	ALP
0	13.35±0.51	12.59±2.01	7.75±0.36	0.09±0.01
50	14.27±1.24	15.70±2.39	6.03±0.41*	0.19±0.07
500	13.05±0.56	16.08±1.33	5.08±0.12**	0.19±0.01**
5000	13.60±1.31	16.68±1.38	5.38±0.33**	0.22±0.01**

Table 12. Effect of insulin upon cell numbers and alkaline phosphatase activity.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released per 30 minutes per 10^6 cells. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, n= 4, * $p < 0.5$, ** $p < 0.01$.

b) Dexamethasone

Dexamethasone significantly decreased the cell numbers ($p < 0.01$) and also caused a significant increase in alkaline phosphatase activity ($p < 0.5$). In cultures supplemented with ascorbic acid (50μg/ml) dexamethasone decreased the cell numbers ($p < 0.01$) but had no effect upon alkaline phosphatase. In cultures supplemented with dexamethasone, ascorbic acid caused a highly significant increase ($p < 0.01$) in both the cell numbers and the alkaline phosphatase activity (Table 13).

Dexamethasone (M)	With ascorbic acid Cell Number	ALP	Without ascorbic acid Cell Number	ALP
0	13.35±0.51	12.59±2.01	7.75±0.36	0.09±0.01
10^{-7}	7.70±0.86**	17.44±0.95	3.18±0.39**	1.35±0.24*

Table 13. Effect of dexamethasone upon cell numbers and alkaline phosphatase activity.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released/30 minutes/10^6 cells. Cell numbers shown as final number x 10^{-5}. Comparing 10^{-7}M with 0M dexamethasone; * $p < 0.05$, ** $p < 0.01$. Mean ± standard error of the mean, n = 4.

v) Effect of growth factors

a) Epidermal growth factor

At no concentration of EGF was there any affect upon the cell numbers. At day 7, EGF caused inhibition of alkaline phosphatase activity (Figure 8). However, by day 14 this effect was no longer present (Table 14).

b) Platelet-derived growth factor

PDGF had an inhibitory effect upon the cell numbers regardless of the presence or absence of ascorbic acid. The effect of PDGF upon alkaline phosphatase activity was not straightforward. In the presence of ascorbic acid PDGF caused a highly significant decrease in the enzyme activity. However, in the absence of ascorbic acid PDGF caused a significant increase.

EGF (ng/ml)	7 days Cell number	ALP	14 days Cell number	ALP
0	11.90±0.96	0.51±0.04	7.60±2.13	6.05±1.87
1	10.05±1.05	0.31±0.02*	11.93±1.02	2.42±0.28
5	12.62±1.18	0.24±0.01**	7.55±1.70	0.52±0.17
10	10.93±1.06	0.23±0.01**	5.35±1.43	0.73±0.32
20	12.15±0.99	0.22±0.02**	2.75±0.81	2.03±0.82

Table 14. Effect of EGF upon cell numbers and alkaline phosphatase activity in the presence of ascorbic acid.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released/30 minutes/10^6 cells. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, n = 4, * $p < 0.05$, ** $p < 0.01$.

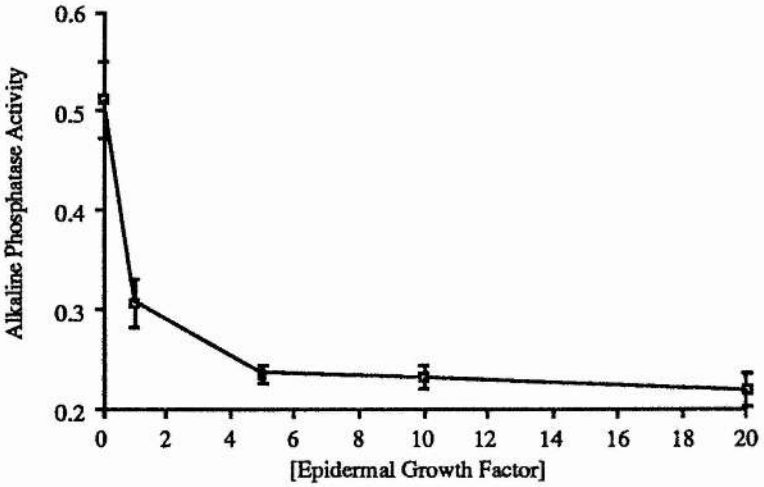
PDGF (ng/ml)	With ascorbic acid Cell numbers	ALP	Without ascorbic acid Cell numbers	ALP
0	7.92±0.77	7.32±0.91	7.75±0.36	0.09±0.01
2	4.85±0.73*	0.27±0.02**	1.75±0.31**	0.29±0.05*

Table 15. Effect of PDGF upon cell numbers and alkaline phosphatase activity.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released/ 30 minutes/ 10^6 cells. Cell numbers shown as final number x 10^{-5}. Comparing with PDGF to without; * $p < 0.05$, * $p < 0.01$. Mean ± standard error of the mean, n = 4.

Figure 8. Graph showing the effect of EGF upon alkaline phosphatase activity.

Alkaline phosphatase activity is expressed as micromoles *p*—nitrophenol released per 30 minutes per 10^6 cells. [EGF] is shown in ng/ml. Mean ± standard error of the mean, n = 4.



5. Microscopy

i) Scanning EM

The surfaces of 14 day cultures of bone cells were found to be covered in large flattened cells upon which were scattered small rounded cells. Large nodular structures were also apparent rising up above the main culture surface. Overlaying images of the X-ray emission of the Kα of calcium onto corresponding micrographs showed distinct localisation of calcium within the nodules suggesting discrete patches of cell proliferation combined with calcium deposition, ie an osteogenic nodule (Figure 9).

A plot of the X-ray emission analysis showed major peaks for phosphorous and the Kα of calcium, with minor peaks for sodium, sulphur and the Kß of calcium (Figure 10).

ii) Transmission EM

At 7 days the cultures had formed multilayers of cells with banded collagen fibres present in the extracellular spaces (Figure 11). The cytoplasm of the cells contained numerous mitochondria and extensive rough endoplasmic reticulum, an indication of active protein synthesis.

At 10 days the collagen fibres within the extracellular spaces were more abundant and in some areas were arranged in a very regular manner. Rough endoplasmic reticulum and mitochondria are still apparent within the cytoplasm of the cultured cells (Figure 12). Matrix vesicles were also present in the cultures and were found in areas of highly disorganised collagen fibres (Figure 13).

At 14 days the cultures contained calcified osteogenic nodules. These consisted of discrete areas of mineral deposited into the extracellular space. The mineralisation front was formed by deposition of spicules of mineral along the collagen fibres perpendicular to the banding pattern (Figure 14). The collagen fibres formed the framework upon which mineral was deposited. The mineral extended up to an unmineralised seam surrounding the cells within the nodule (Figure 15). In the centre of the nodule there were cells completely surrounded by mineral (Figure 16). These cells resembled osteocytes very closely. Cells

on the surface of the multilayered cultures were rounded up and the cytoplasm contained mitochondria but very little rough endoplasmic reticulum (Figure 17).

iii) Histology

Confluency was reached 2 to 3 days following seeding with the cells exhibiting a polygonal morphology (Figure 18). The cultures rapidly developed multilayers with areas of nodule formation. In the absence of ß-glycerophosphate there was no calcium deposition whereas with ß-glycerophosphate calcification was localised to within the nodules (Figures 19a and 19b).

Figure 9. (Mag. x 400)

Transmission electron micrograph of the surface of a 14 day old primary culture of neonatal mouse calvarial osteoblasts showing an osteogenic nodule (N). A speckled image of the X-ray emmission of the Kα of calcium was overlaid, showing the localisation of calcium to the nodule.


N

Figure 10.

A plot of the X-ray emmission analysis of a mineralised osteogenic nodule. There are prominent peaks for phosphorous (P) and calcium (Kα), with small peaks for sodium (NA), sulphur (S) and calcium (Kß).

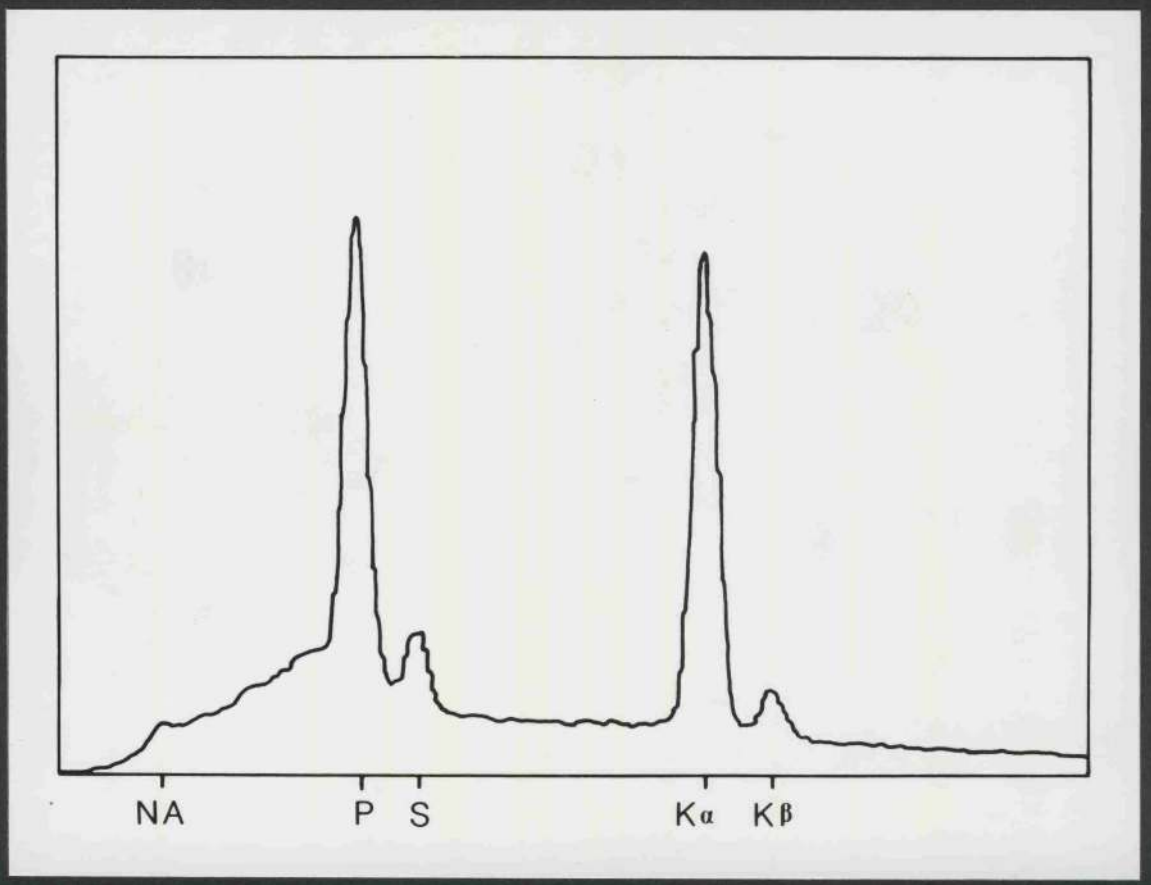

NA
P
S
Kα
Kβ

Figure 11. (Mag. x 30,700)

Transmission electron micrograph from a 7 day old culture.

Figure 12. (Mag. x 28,170)

Transmission electron micrograph from a 10 day old culture.

The cultures have started to form multilayers of cells. The cytoplasmic organelles are characteristic of cells involved involved in active protein synthesis; mitochondria (MC) and rough endoplasmic reticulum (RER). The extracellular spaces contains collagen fibrils in transverse (TS) and longitudinal section (LS).


MC
RER
TS
LS

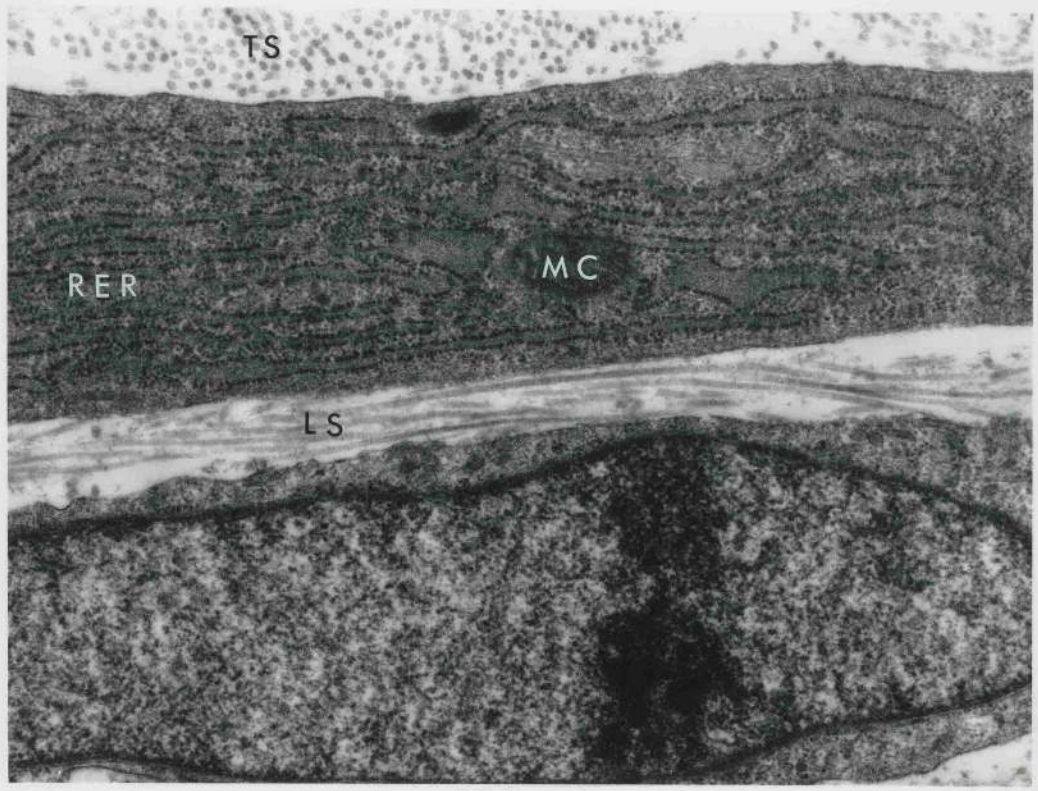

TS
RER
MC
LS

Figure 13. (Mag. x 42,650)

Transmission electron micrograph from a 10 day old culture. The extracellular space contains abundant collagen fibrils in transverse (TS) and longitudinal section (LS). The collagen fibrils appear to be randomly arranged and are interspersed with matrix vesicles (MV).

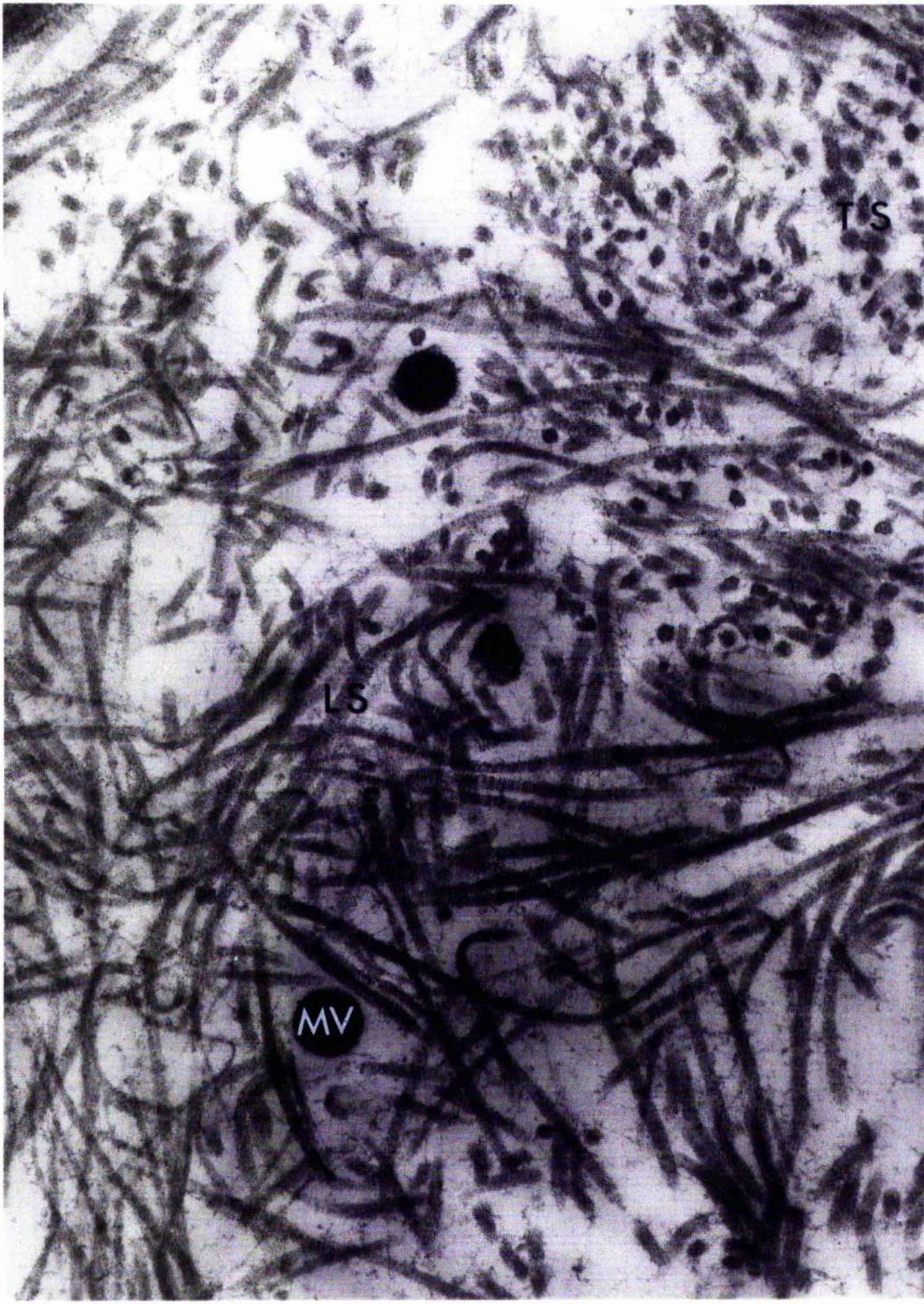

TS
LS
MV

Figure 14. (Mag. x 56,350)

Transmission electron micrograph from a 14 day old culture. Mineral (M) has been deposited on a framework provided by the collagen fibrils (C). Spicules (S) of mineral are extending along the fibrils and lie parallel to the main axis of the fibril. Transverse banding (B) with a periodicity of approximately 62nm is apparent on the fibrils.


C
B
S
M

Figure 15. (Mag. x 67,050)

Transmission electron micrograph from a 14 day old culture. The extracellular space has been filled with mineral (M). The osteoblasts is still capable of protein synthesis, as shown by the presence of mitochondria (MC) and rough endoplasmic reticulum (RER). Between the cell and the mass of mineral lies an unmineralised seam of osteoid (O).

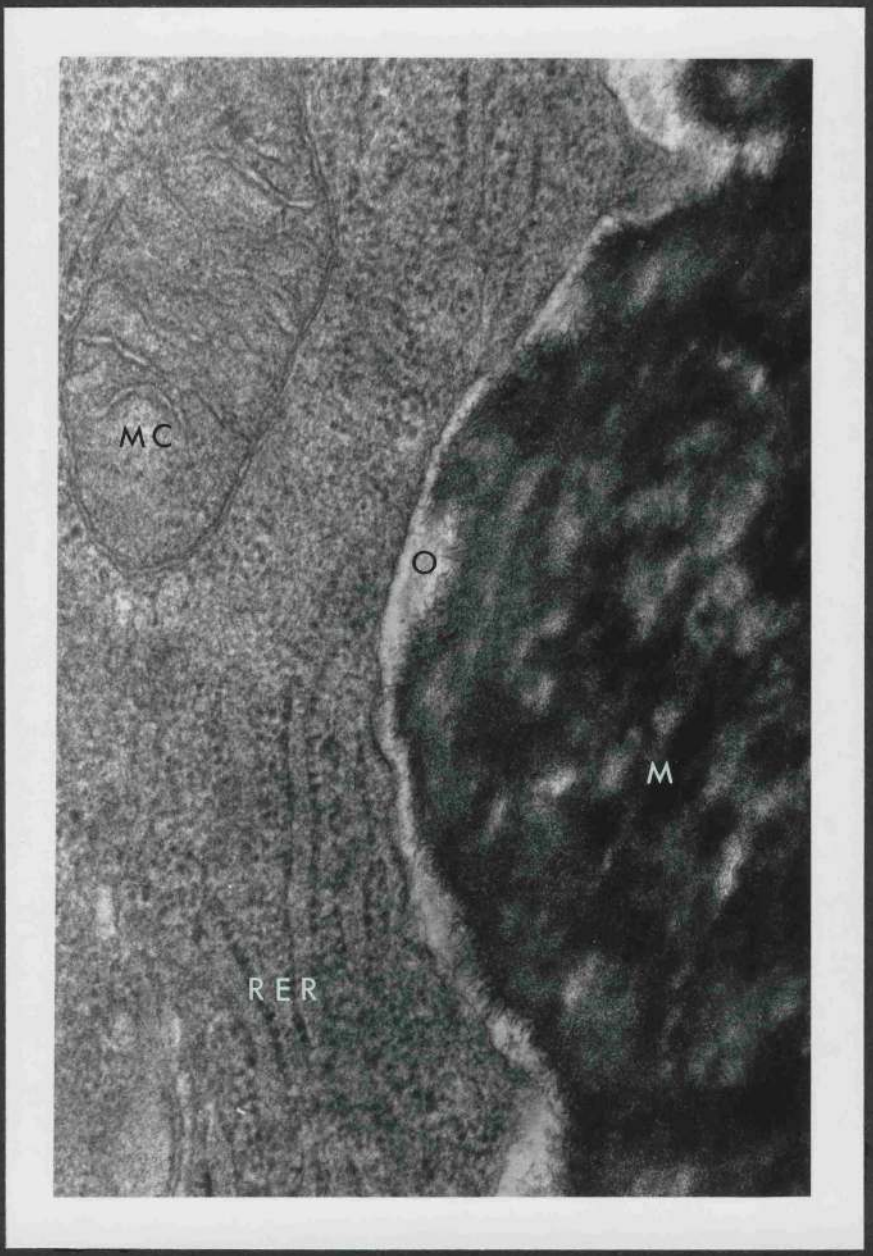

MC
O
M
RER

Figure 16. (Mag. x 10,820)

Transmission electron micrograph from a 14 day old culture. An osteocyte (OC) has become completely surrounded by mineral (M). An unmineralised seam of osteoid (O) is apparent, separating the cell from the mineral. The cell is extending processes (P) towards the mineral, one of which is lying in a canaliculum.

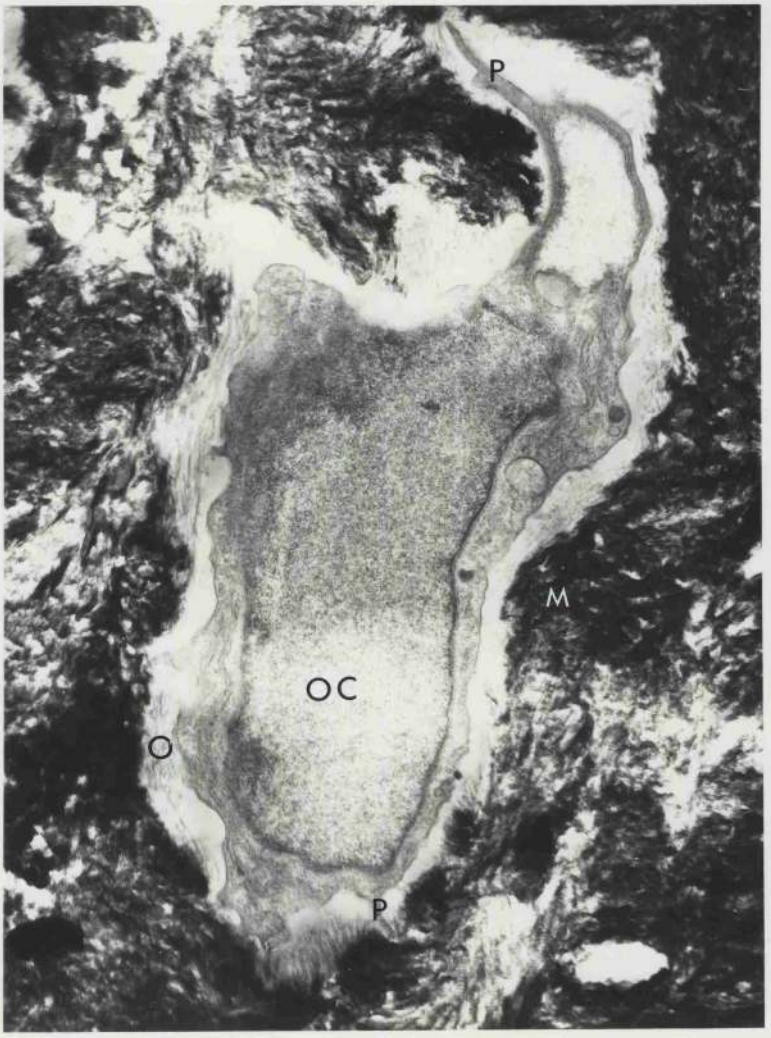

P
M
OC
O
P

Figure 17. (Mag. x 14,250)

Transmission electron micrograph from a 14 day old culture. A rounded cell lying on the surface of a nodule. The cytoplasm contains mitochondria (MC) and some rough endoplasmic reticulum (RER).

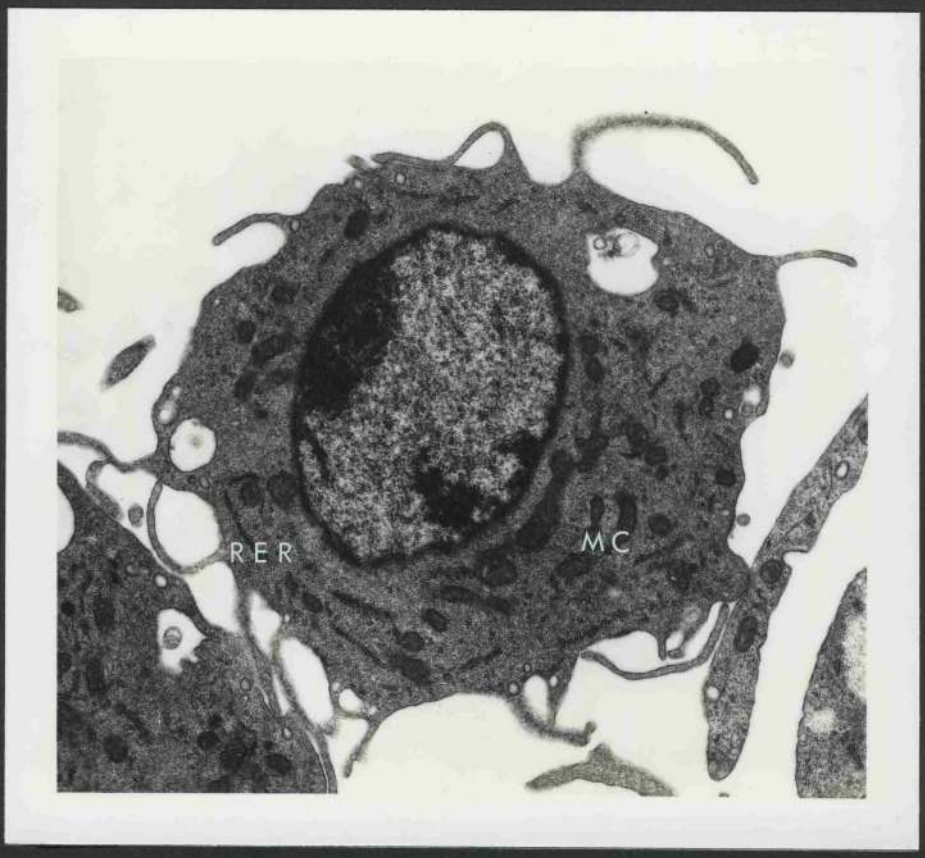

RER
MC

Figure 18. (Mag. x 140)

Phase contrast light micrograph of a culture of 2 to 3 day old primary osteoblasts. The cells have formed a monolayer of polygonal cells.

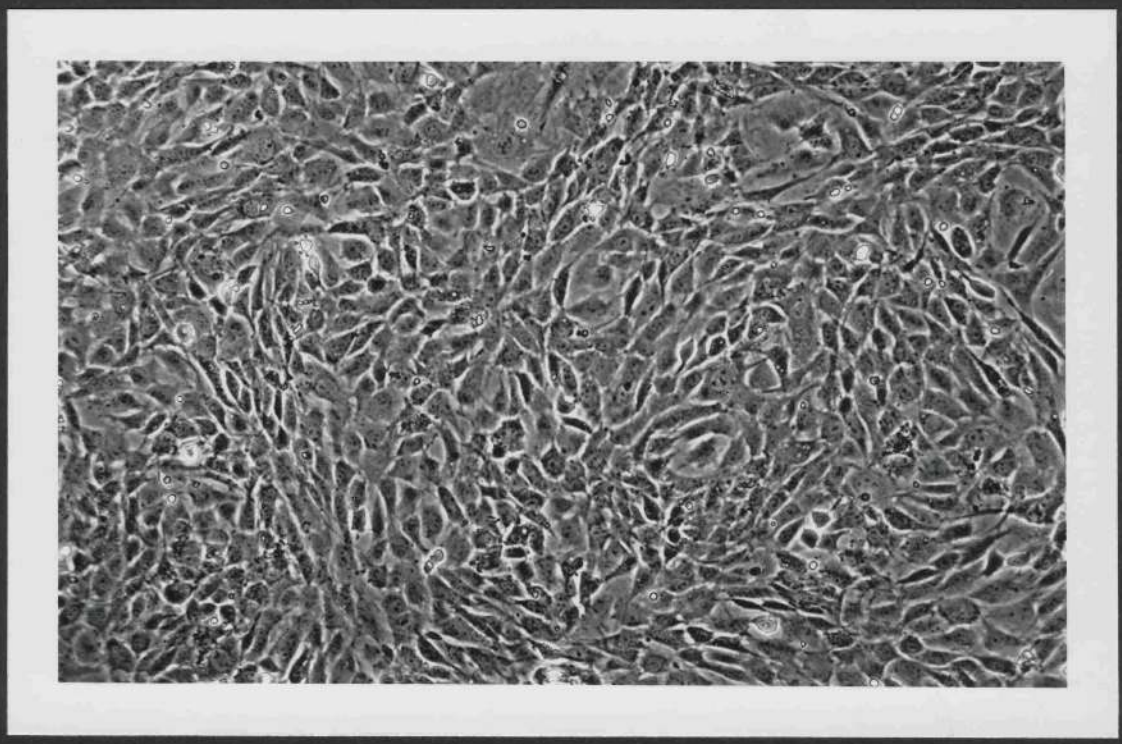
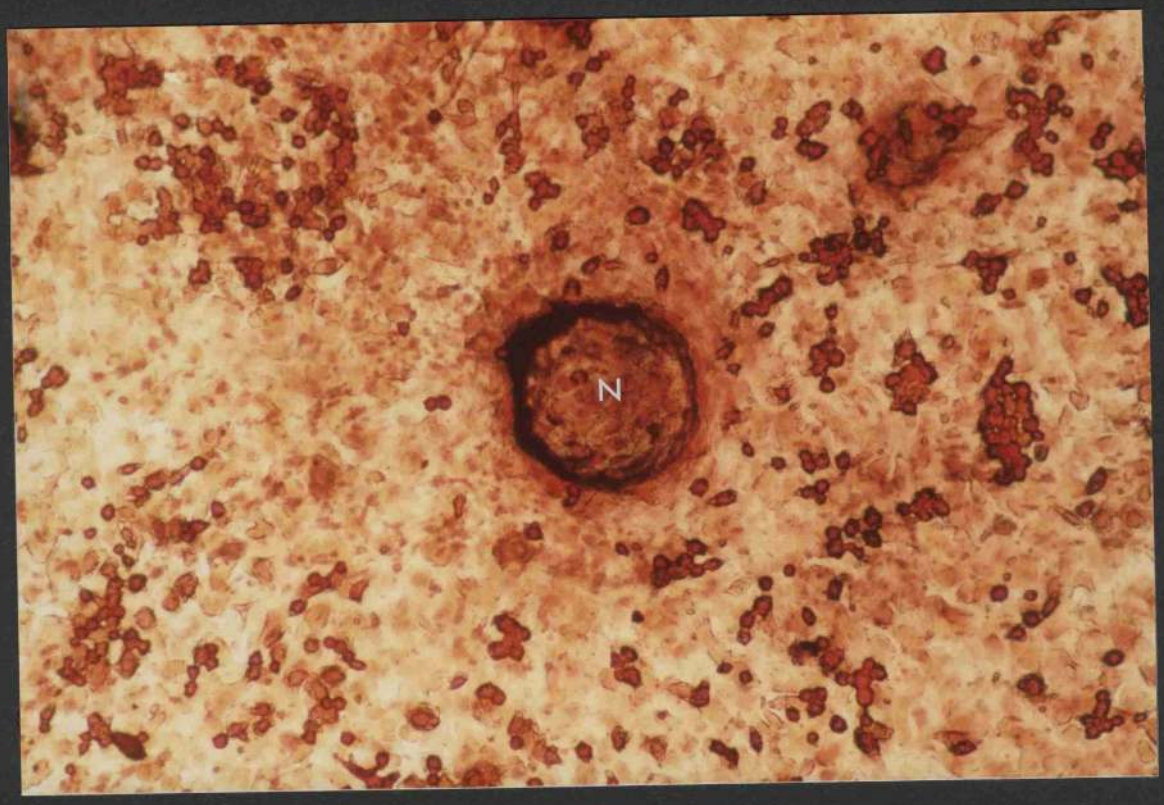


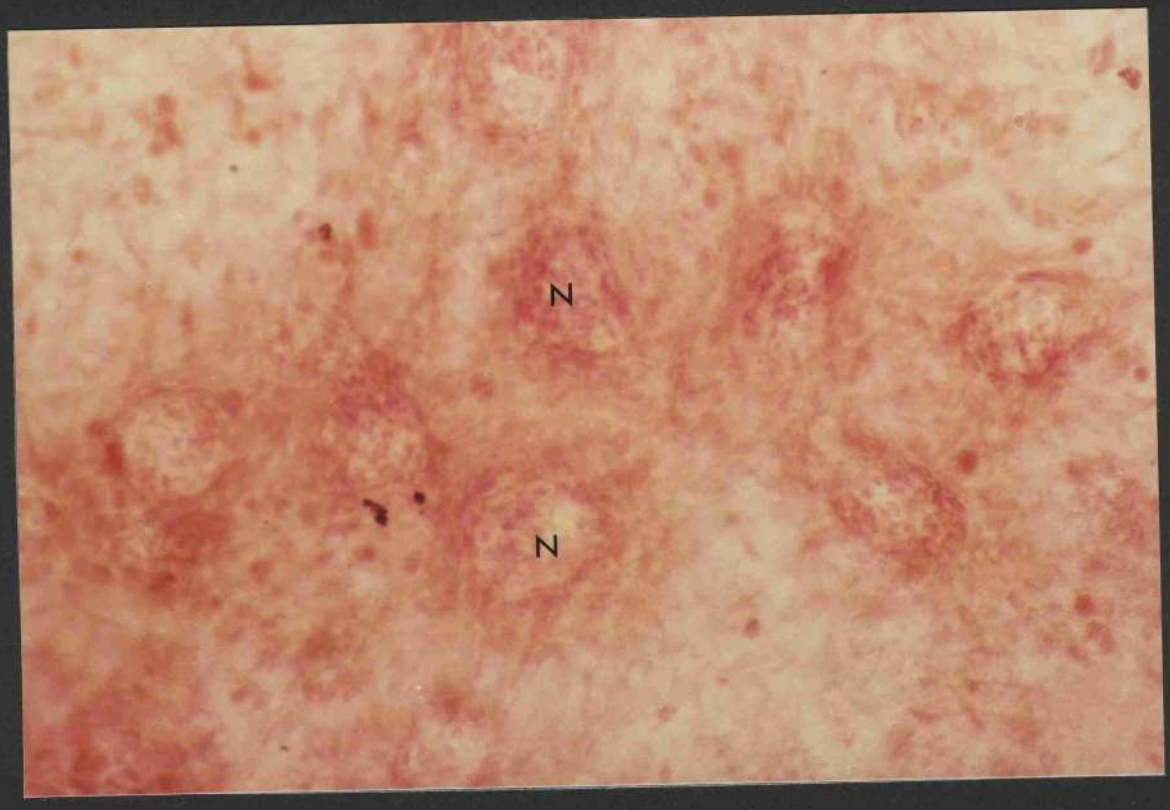
Figure 19a. (Mag. x 160)

Light micrograph of osteoblasts cultured with ascorbic acid and without ß-glycerophosphate. Osteogenic nodules have formed (N). However, these are unmineralised due to the lack of phosphate.

Figure 19b. (Mag. x 160)

Light micrograph of osteoblasts cultured with ascorbic acid and ß-glycerophosphate. An osteogenic nodule (N) has formed and has mineralised. Insoluble calcium is shown by the presence of black deposits of silver.


N


N
N

6. Mineralisation *in vitro*

i) Effect of hormones

a) Insulin

At low concentrations of insulin, no calcified matrix formed in the absence of ascorbic acid. However, patches of mineral appeared in cultures supplemented with 5000ng/ml insulin. The percentage of the area mineralised was minimal; 0.5±0.2 (n = 4).

In the presence of ascorbic acid, insulin caused a significant increase ($p < 0.05$) in the area of calcification at 500ng/ml, and a highly significant increase ($p < 0.01$) at 5000ng/ml (Figure 20).

b) Dexamethasone

In the presence of ascorbic acid, dexamethasone caused a marginally significant decrease ($p = 0.05$) in the area of calcification (Table 16).

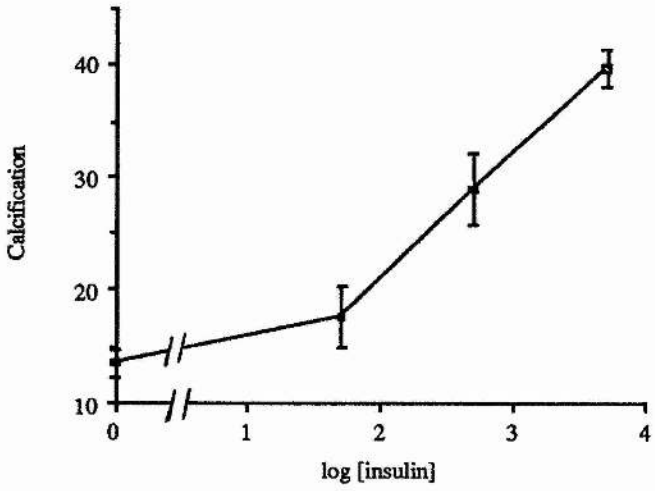
Dexamethasone (M)	Calcification
0	13.6±1.4
10^{-7}	7.2±0.3*

Table 16. Data showing the effect of dexamethasone upon the extent of calcification.

Extent of calcification expressed as a percentage of the total area. Mean ± standard error of the mean, n = 4. *, $p = 0.05$.

Figure 20. Graph showing the effect of insulin upon the extent of mineralisation.

Calcification expressed as a percentage of the total area. [Insulin] shown as the log (ng/ml). Mean ± standard error of the mean, n = 4.



ii) Effect of growth factors

a) Epidermal growth factor

EGF caused a highly significant ($p < 0.01$) decrease in calcification at all concentrations tested (Figure 21).

b) Platelet-derived growth factor

In the presence of PDGF (2ng/ml) there was no observable calcification. The percentage of the total area calcified in control cultures was 3.7±0.8 (mean ± standard error of the mean, n = 4).

7. Quantitative calcium assay

i) Assay calibration

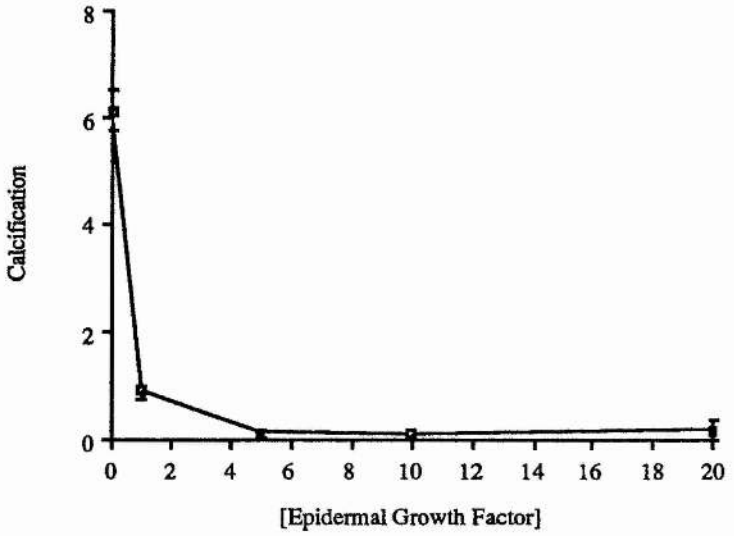
The correlation between the change in absorbance and the concentration of soluble calcium was linear within the range tested.

ii) Effect of organic phosphate

Addition of organic phosphate caused a significant increase in calcium deposited at 5mM and a highly significant increase at 10mM. There was no effect upon cell numbers (Table 17).

Figure 21. Graph showing the effect of epidermal growth factor upon the extent of calcification.

Calcification expressed as a percentage of the total area. [EGF] shown as ng/ml. Mean ± standard error of the mean, n = 4.



ß-glycerophosphate (mM)	Cell numbers	Calcium content
0	7.25±0.65	0.8±0.3
1	8.43±0.35	1.1±0.1
5	7.03±1.10	33.2±9.3*
10	5.97±0.66	51.1±7.4**

Table 17. Effect of organic phosphate upon cell numbers and calcium content.

Calcium content expressed as $\mu g/10^6$ cells. Cell numbers shown as final number x 10^{-5}. Mean ± standard error of the mean, n = 4, * $p < 0.05$, ** $p < 0.01$.

iii) Effect of seeding density

The cell concentration at seeding had a highly significant effect upon the cell density at the end of the culture period. Despite this, however, there was little significant effect upon the dry weight of each sample, suggesting that the measurement of the dry weight of the samples was not as accurate and reliable as counting the cell numbers. The amount of calcium deposited was significantly increased by increasing the seeding density (Table 18).

Seeding ($x10^5$)	Dry weight (mg)	Cell number	Calcium (μg/mg dry weight)	Calcium ($\mu g/10^6$ cells)
0.1	7.7±0.2	3.68±0.45	0.1±0.1	2.4±1.5
0.5	8.8±0.4	13.50±0.39	2.0±0.3*	12.7±1.5**
1.0	8.5±0.1	19.55±0.87	5.8±0.1**	25.6±4.4*
2.0	7.9±0.6	22.08±1.60	3.8±1.1*	13.2±3.4*
4.0	8.5±0.2	26.05±0.77	5.2±1.5*	17.4±5.6

Table 18. Effect of seeding density upon calcium deposition.

Cell numbers shown as final cell number x 10^{-5}. Mean ± standard error of the mean, n = 4, * $p < 0.05$, ** $p < 0.01$.

8. Granulocyte/macrophage colony forming cell assay

Cultures plated without WEHI-3 or bone cell conditioned medium produced no colonies. Parallel cultures supplemented with WEHI-3 produced 38.0 ± 5.8 colonies (mean ± standard error of the mean, n = 4).

Addition of bone cell conditioned medium to the cultures resulted in the stimulation of colony formation due to the presence of a colony stimulating factor (Figure 22). CM from older osteogenic cultures contained more CSF, as shown by the increase in colony formation.

The proportion of colony types was initially no different from that found in WEHI-3 stimulated cultures (Table 19). From day 4 to 8 the percentage of loose colonies formed was significantly less than that in WEHI-3 stimulated controls. The percentages of mixed and tight colonies were increased over controls. This indicates the transient presence of some factor other that GM-CSF, possibly M-CSF.

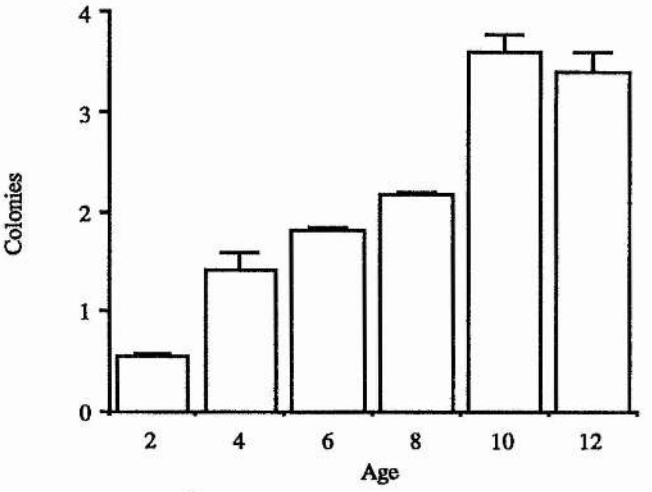
Colony type	Days 2	4	6	8	10	12
Loose	17.4±10.0	6.1±34.4**	75.9±4.8*	83.6±1.8*	98.07±2.3	89.8±2.3
Mixed	31.7±8.9	11.3±9.4	23.7±4.0*	16.4±1.8*	1.7±0.7	9.2±1.3
Tight	50.9±13.0	82.6±10.0*	-	-	-	-

Table 19. Table showing the percentage of colony types following stimulation with bone cell conditioned medium.

Loose - colonies of macrophages. Tight - colonies of granulocytes. Mixed - colonies of both macrophages and granulocytes. The age of the bone cell cultures at the time of conditioned medium collection is shown in days. Mean ± standard error of the mean, n = 4. Comparing experimental cultures with cultures supplemented withWEHI-3 (GM-CSF), * $p < 0.05$, ** $p < 0.01$.

Figure 22. Graph showing the effect of culture age upon the ability of the conditioned medium produced to stimulate colony formation.

Colonies shown as the number of colonies formed relative to WEHI-3 stimulated controls. This is plotted against the age of the bone cell culture when the conditioned medium was harvested. Mean ± standard error of the mean, n = 4.



9. Balb/c 3T3 cell line

i) Conditioned medium

Conditioned medium was collected from osteogenic cultures of neonatal mouse osteoblasts. Following concentration by dialysis, the total protein content of the concentrated conditioned medium was found to be 13.7mg/ml.

ii) Alkaline phosphatase assay

At no concentration tested did supplementation with bone cell conditioned medium have any effect upon the expression of alkaline phosphatase by 3T3 cells (Table 20).

iii) Protein content

There was no effect of CM upon the protein content of cultured 3T3 cells expressed as μg total protein/10^6 cells. At 10μg/ml, CM caused a marginally significant decrease in 3T3 cell numbers, rising to a highly significant increase in cell numbers at 500μg/ml.

iv) Calcium deposition

Any calcium deposited by the 3T3 cells was in such small quantities as to be undetectable using the standard assay method.

CM (μg/ml)	Cell Number	Alkaline Phosphatase
0	8.70±0.38	0.06±0.01
10	7.28±0.39*	0.07±0.01
25	8.70±0.21	0.06±0.00
50	8.93±0.12	0.06±0.00
100	9.60±0.39	0.07±0.01
500	13.65±0.46**	0.05±0.00

Table 20. Effect of bone cell conditioned medium upon Balb/c 3T3 cells.

Alkaline phosphatase activity expressed as μmoles *p*-nitrophenol released/30 minutes/10^6 cells. Cell numbers shown as final cell number x 10^{-6}. Mean ± standard error of the mean, n = 4, * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

LIST OF CONTENTS

1. Osteoblasts *in vitro* 107
   - i) Methods of isolation 107
   - ii) Viability 109
   - iii Expression of the osteoblastic phenotype 110

2. Expression of alkaline phosphatase *in vitro* 110
   - i) Effect of ascorbic acid 111
   - ii) Effect of organic phosphate 112
   - iii) Effect of hormones 113
     - a) Insulin 113
     - b) Dexamethasone 114
   - iv) Effect of growth factors 116
     - a) Epidermal growth factor 116
     - b) Platelet-derived growth factor 116

3. Osteogenesis *in vitro* 117
   - i) Morphology 117
   - ii) Effect of organic phosphate 119
   - iii) Effect of seeding density 119
   - iv) Effect of hormones 119
     - a) Insulin 119
     - b) Dexamethasone 120
   - v) Effect of growth factors 120
     - a) Epidermal growth factor 120
     - b) Platelet-derived growth factor 121

4. Osteoblastic products *in vitro* 121
   - i) Haemopoietic cell growth factors 121
   - ii) Effect on mesenchymal cells 123

1. Osteoblasts *in vitro*

i) Methods of isolation

Three different methods were used to obtain populations of osteoblasts from neonatal mice. Briefly, method I was used to determine the distribution of alkaline phosphatase positive cells within the calvarial tissues. Method II followed a more involved digestion procedure and was used to assess the ability of two populations, over a range of neonatal ages, to express alkaline phosphatase in culture. Method III used the same enzymatic regimen (except for the lack of trypsin) as method II. The rate of rotation of the magnetic flea agitating the digestion mixture was much slower and consequently the rate of release of cells from the calvaria was greatly delayed. The cells released were pooled to form one population and this provided the basis for all subsequent experimental work involving bone cell cultures.

The data in Table 5 show the percentages of alkaline phosphatase positive cells recovered from the periosteum, sutures and the calvaria. The sutures contained very few osteoblasts, but this is to be expected as they are fibrous connective tissue structures containing large numbers of fibroblasts (Yagiela and Woodbury, 1977). The sparsity of osteoblasts within the periosteum was surprising since this tissue has been shown to have a high osteogenic potential *in vitro* (Tenenbaum and Heersche, 1982). However, this may be due to;

i) the osteogenic cells within the periosteum belonging to the osteoprogenitor compartment, and therefore probably alkaline phosphatase negative, or
ii) a species difference between mice (used in this study) and chicks (used by Tenenbaum and Heersche, 1982), or
iii) incomplete removal of the periosteum leaving a basal osteogenic layer still attached to the calvaria, or
iv) damage and death of osteoblasts during periosteal removal.

The populations derived from the calvaria showed an increasing abundance of osteoblasts as the digestion procedure progressed, reaching a peak in the third population. This is in agreement with Wong and Cohn (1975) who found a peak of cAMP responsiveness to PTH in populations 4 and 5, out of 6 in total. Population I obviously contains a large proportion of non-osteoblastic cells and these may well be cells associated with the blood vessels, bone lining cells and fibroblastic remnants of the periosteum. This first collagenase digestion could therefore function as a 'cleaner', freeing a large number of non-osteoblastic cells from the calvaria. The subsequent populations (II, III and IV) contained significantly more osteoblasts, suggesting that these digestions are removing cells directly associated with the bone. The alkaline phosphatase negative cells may represent fibroblasts, osteoprogenitors and cells derived from the developing marrow (Yagiela and Woodbury, 1977).

Method II was based on that used by Heath and colleagues (1984). The periosteum was left intact and the saggital suture was removed by careful dissection. Even though the periosteum contained few osteoblasts, it was felt that mechanically stripping away the membrane would damage underlying osteoblasts. The calvaria were first digested with trypsin for 10 minutes, at which time the supernatant was discarded and replaced with dispase. The cells released during this period were termed the early population. The pooled cells from the three subsequent collagenase digestions were termed the late population.

Both populations of bone cells grew well in culture (Tables 6a and 6b). However, the early population had a greater rate of proliferation, and this may reflect a substantial fibroblastic component, possibly derived from the periosteum. Also, both populations showed an initial drop in alkaline phosphatase followed by a rise over the time course of the experiment. The rise was such that by day 5 of cultures of the late population, the amount of alkaline phosphatase was not significantly different from that at day 1. This can be explained by regarding each population as being composed of two sub-populations, osteoblastic and non-osteoblastic, although this is probably an over simplification.

The non-osteoblastic cells of the early population were in the minority at seeding. However, they were highly prolific and quickly over-ran the slower growing osteoblasts. The rise in the relative proportion of osteoblasts at day 5 may represent the start of the log phase of growth of these cells as the non-osteoblastic cells started to plateau. The non-osteoblastic cells in the late population appeared to be less prolific and the proportion of osteoblasts started to rise at an earlier stage. This may be due to the non-osteoblastic cells of the late population belonging to a more differentiated compartment than those of the early population, and consequently possessing a reduced proliferative potential.

Interestingly, comparison between parts 2ii) and 2iii) shows an increase in the proportion of osteoblasts extracted, although ostensibly using the same method. All things being equal, this may reflect an increase in technical standard and care during handling of the tissue.

Method III was basically very similar to method II. However, the initial trypsin digest was absent and, more importantly, the rate of agitation of the digestion mixture (by a magnetic flea) was much slower. This resulted in a delayed release of cells. The dispase digest was used to remove periosteal cells which were assumed to have a correlation with the highly proliferative, non-osteoblastic cells in population I (method I) and the early population (method II).

ii) Viability

The viability of the cells released was assessed solely for enzymatic digestion following method II (Table 3). Viability was determined by measuring the ability of the cells to exclude trypan blue, as it was assumed that only live cells were capable of excluding the dye. The first two populations of cells released contained very few cells, a large majority of which were unviable. The sparsity of cells is probably due to the shortness of the digestion periods. These initial populations represent a wash and short digest of the calvarial surfaces and would, therefore, be expected to contain blood cells, non-adherent dead cells and cells damaged during dissection by exposure to air and by the physical actions of dissection.

The four subsequent 30 minute digestions contained large numbers of cells and had a percentage viability of ≥95%. The high viability and cell yield suggested that these populations would be suitable for establishing cultures of bone cells. The third population (dispase) was seeded as the **early** population and populations 4 to 6 were seeded as the **late** population.

iii) Expression of the osteoblastic phenotype

It has been seen that the bone cells were capable of expressing alkaline phosphatase following a period of growth *in vitro* (Tables 6a and 6b) and that this is a characteristic of the osteoblastic phenotype. Bone cells *in vitro* can also be characterised by their ability to produce cAMP in response to hormonal stimulation by both PTH and CT (Rodan and Rodan, 1974; Wong and Cohn, 1974). It is different populations, however, acting as the target cell for each hormone (Wong and Cohn, 1975). Biochemical characterisation of the two target cell types led to the conclusion that response to PTH by cAMP production indicated a cell population enriched for osteoblasts. The other cell population responded to both PTH and CT and it was thought to consist of osteoclasts and osteocytes (Luben *et al.*, 1976). The osteoblastic nature of the PTH-responsive cells is given further credence by the predominance of this cell type in periosteum-free calvaria (Peck *et al.*, 1977). The response to PTH and CT within the assay system employed here is therefore characteristic of a strong osteoblastic presence in the cell population.

2. Expression of alkaline phosphatase *in vitro*

Although the role of alkaline phosphatase in bone is unclear, the expression of this enzyme is characteristic of the osteoblastic phenotype (Rodan and Rodan, 1984). It is reasonable, therefore, to use this as a marker of osteogenic differentiation in cultures of bone cells. Two methods were used to assay for alkaline phosphatase; a histochemical and a quantitative method. Histochemical localisation of alkaline phosphatase provided information concerning the proportion of enzyme-positive cells, whereas the quantitative method gave a measure of the enzyme activity.

i) Effect of ascorbic acid

Bone cells cultured for up to 14 days in unsupplemented growth medium (controls) showed a general decline in alkaline phosphatase levels. The effect of ascorbic acid was very pronounced causing a highly significant increase in alkaline phosphatase expression, and this implied a profound role in osteogenic differentiation (Figure 4). Ascorbic acid has also been shown to have significant stimulatory effects upon alkaline phosphatase in short-term cultures of an osteoblast cell line, UMR-106 (Sugimoto *et al.*, 1986).

Initially, ascorbic acid appeared to have no affect upon alkaline phosphatase expression. However, by day 4 the level of enzyme expression started to rise and then plateau. This was an extension of the phenomenon seen in Tables 6a and 6b and would have the same underlying cause. The prolonged time course shows that a state was reached in which the proportion of alkaline phosphatase positive cells was relatively steady, indicating that the osteoblastic and non-osteoblastic populations reached an equilibrium.

Ascorbic acid was found to have no inhibitory effect upon proliferation, indeed at 50μg/ml there was a significant increase over the 14 day culture period (Table 8). However, at day 14, a Student's t test showed that various concentrations of ascorbic acid had no effect upon cells numbers over control levels (Table 9). Ascorbic acid has been shown to be toxic under aerobic conditions due to the formation of hydrogen peroxide (Koch and Biaglow, 1978);

$$\text{ascorbic acid} + O_2 \dashrightarrow H_2O_2 + \text{dehydroascorbic acid}$$

Under hypoxic conditions, ascorbic acid is not toxic. The lack of toxicity due to the action of ascorbic acid in this system was possibly due to the ability of the osteoblastic cells to form multi-layers very quickly following confluence. In this state, only the uppermost cells would experience maximally aerobic conditions and the underlying cells would be protected against the toxicity of ascorbic acid by the relative lack of oxygen. This is, however, very speculative.

Ascorbic acid caused a significant increase in alkaline phosphatase activity at day 7, however, this disappeared by day 14 (Table 10). As has been seen, the proportion of cells

expressing alkaline phosphatase is increased significantly by ascorbic acid (Figure 4). Therefore if the percentage of alkaline phosphatase positive cells has increased while there is no change in the enzyme activity then the activity per alkaline phosphatase positive cell has decreased. This may represent a decline in activity as osteoblasts differentiate into osteocytes. Figure 7 shows the gradual increase and the decline in alkaline phosphatase with time in culture. Ascorbic acid had no effect upon cell numbers at two time points (7 and 14 days), indicating a lack of any toxic action.

These results show a pronounced effect of ascorbic acid upon osteoblasts: the vitamin is required for maintenance of alkaline phosphatase levels. Ascorbic acid is also necessary for the production of collagen (de Clerk and Jones, 1980). In the absence of ascorbic acid, under-hydroxylated collagen is produced (Pinnell *et al.*, 1987). Ascorbic acid has also been shown to stimulate both alkaline phosphatase expression and collagen synthesis in primary cultures of embryonic chick chondrocytes (Habuchi *et al.*, 1985). An approximation to the full expression of the osteoblastic phenotype *in vitro* is therefore dependent upon the addition of ascorbic acid to the culture medium.

ii) Effect of organic phosphate

The decline in alkaline phosphatase levels apparent in control cultures was delayed, but not prevented, by the addition of organic phosphate to the culture medium (Figure 5). However, the delay was such that ß-glycerophosphate caused a highly significant increase in alkaline phosphatase expression over control levels. In the presence of ascorbic acid, however, ß-glycerophosphate caused a significant decrease in alkaline phosphatase levels (Table 7). Although by day 14 this was no longer significant as organic phosphate had no effect upon cell numbers or alkaline phosphatase activity over a range of concentrations (Table 11).

Other studies have shown that ß-glycerophosphate can cause a significant decrease in alkaline phosphatase activity. Tenenbaum and colleagues (1986) found that 10mM ß-glycerophosphate inhibited alkaline phosphatase activity by day 6 in explants of embryonic chick periostea in the presence of ascorbic acid. Ashton and colleagues (1986) also found

inhibition of alkaline phosphatase activity by day 6 in passaged cells grown out from fragments of human bone in the presence of ascorbic acid. Therefore ß-glycerophosphate stimulates alkaline phosphatase levels in the absence of ascorbic acid, and inhibits alkaline phosphatase in the presence of ascorbic acid. The inhibition is transient, however, as it has disappeared by day 14.

ß-glycerophosphate can act as a substrate for alkaline phosphatase and is hydrolysed to release phosphate (Stinson and Chan, 1987). However, phosphate is a competitive inhibitor of alkaline phosphatase, due probably to its structural resemblance to ß-glycerophosphate. Therefore, in the absence of ascorbic acid, alkaline phosphatase levels are not elevated and the ratio of ß-glycerophosphate to alkaline phosphatase is higher than it is in the presence of ascorbic acid. The high comparative concentration of ß-glycerophosphate will therefore overcome inhibition due to phosphate, and indeed, alkaline phosphatase levels are increased.

In the presence of ascorbic acid, alkaline phosphatase activity is stimulated and therefore the ratio of ß-glycerophosphate to alkaline phosphatase is lowered, allowing inhibition by phosphate. However, in prolonged culture with ascorbic acid the level of alkaline phosphatase activity reaches a plateau, therefore as ß-glycerophosphate is added to the culture at media changes the ratio of ß-glycerophosphate to alkaline phosphatase will rise and inhibition due to phosphate will be overcome, ie inhibition is transient and disappears by day 14.

iii) Effect of hormones

a) Insulin

Insulin has also been shown to stimulate cell proliferation in cultures of neonatal rat bone cells and UMR-106 cells, a rat osteosarcoma cell line (Schmid *et al.*, 1984; Hickman and McElduff, 1989). The primary cultures of rat osteoblasts were incubated with serum-free medium containing insulin from day 1 to day 7. This caused an increase in the cell numbers and alkaline phosphatase activity. The UMR-106 cells were exposed to insulin for 20 hours following confluence, and this caused an increase in cell replication. By day

14, however, there was inhibition of replication in mouse osteoblasts cultured in the absence of ascorbic acid (Table 12). This may be due to an initial stimulation of division with a subsequent depletion of the proliferative pool.

Insulin stimulates alkaline phosphatase activity in cultures of neonatal rat osteoblasts and organ cultures of fetal rat calvaria (Schmid *et al.*, 1984; Canalis, 1983a). However, insulin has also been shown to inhibit alkaline phosphatase activity in a rat osteosarcoma cell line, ROS (Levy *et al.*, 1986). In this study, neonatal mouse osteoblasts responded to insulin (and only in the absence of ascorbic acid) with an increase in alkaline phosphatase activity by day 14 in culture (Table 12). The inhibition of alkaline phosphatase activity in cultures of ROS cells only occurred at sub-confluence. Insulin had no effect upon alkaline phosphatase activity at confluence. It would seem, therefore, that in the absence of ascorbic acid, insulin first inhibits and then stimulates alkaline phosphatase activity in sub-confluent and post-confluent cultures respectively. The absence of an insulin-induced effect in the presence of ascorbic acid maybe due to swamping, as the stimulation caused by 5000ng/ml insulin is a very small fraction of that caused by 50μg/ml ascorbic acid (Table 12). The effect of insulin (but only in the absence of ascorbic acid) is to inhibit proliferation while stimulating differentiation.

b) Dexamethasone

Over the 14 day culture period, dexamethasone (10^{-7}M) had a significant stimulatory effect upon alkaline phosphatase levels in the presence of ascorbic acid and ß-glycerophosphate (Table 7). In short-term cultures (up to 96 hours), dexamethasone (10^{-9} to 10^{-5}M) and other glucocorticoids have been shown to cause a transient stimulation of alkaline phosphatase expression followed by inhibition (Canalis, 1983b). However, Canalis (1983b) studied this aspect of osteoblastic behaviour in organ cultures of fetal rat calvaria. Organ cultures contain all the cell types associated with bone and also maintain the microenvironment. Dexamethasone may be exerting a direct action upon preosteoblasts and osteoblasts, causing the initial increase in alkaline phosphatase activity. The cultures of isolated osteoblasts used in this study responded to dexamethasone with a sustained

increase in alkaline phosphatase levels. However, organ cultures of calvaria will contain osteo-progenitors and the eventual decline of alkaline phosphatase may be explained by dexamethasone inhibiting progenitor differentiation and proliferation and consequently halting progression through the osteogenic lineage. Another glucocorticoid, cortisol, has been shown to affect bone growth by primarily inhibiting the proliferation of osteoprogenitors (Chyun *et al.*, 1984). Dexamethasone may also stimulate the progenitor cells in organ cultures to produce a factor inhibiting preosteoblast and osteoblast differentiation. So, even though osteoblast cultures are depleted in progenitors, dexamethasone would cause an overall stimulation of alkaline phosphatase in the absence of any inhibiting factor(s) produced by dexamethasone-induced stimulation of osteo-progenitors.

Dexamethasone had no effect upon proliferation (Table 8). In other studies, the effects of dexamethasone upon proliferation have been varied, depending upon the age and type of culture and the species involved. Proliferation of rat osteoblasts is inhibited by dexamethasone in sparse and sub-confluent cultures (Chen *et al.*, 1983; Chen and Feldman, 1979). There is no effect upon cultures of intermediate cell density and cultures treated at a post-confluent stage. However, dexamethasone stimulates proliferation in dense cultures. In organ cultures, there is no effect at 24 hours, but by 96 hours there is inhibition of proliferation (Canalis, 1983b). In chick periosteal explants, dexamethasone stimulates proliferation up to 6 days in culture (Tenenbaum and Heersche, 1985). Dexamethasone inhibits proliferation in cultures of mouse osteoblasts at all densities (Chen *et al.*, 1983).

The inhibition of proliferation seen in the cultures of mouse osteoblasts by Chen and colleagues (1983) cannot be directly compared with the lack of effect seen in this study. The incubation period was over 5 days and, perhaps more importantly, the culture medium lacked ascorbic acid and organic phosphate. In the absence of ascorbic acid, the proportion of alkaline phosphatase positive cells will be reduced and would therefore be a different cell population to that used in this study. Dexamethasone may exert differential

effects upon alkaline phosphatase negative and positive cells, causing an overall inhibition in the experiments of Chen and colleagues and no effect in this study.

Dexamethasone caused a highly significant decrease in cell numbers at day 14 (Table 13). As no inhibition was seen over the 14 day period as a whole (Table 8), this may represent a delayed decrease in proliferation due to inhibition of osteoprogenitor differentiation. Alkaline phosphatase activity is stimulated in osteoblasts cultured in the absence of ascorbic acid (Table 8). However, even though significant, the stimulation is slight and may be masked by the more intense stimulation caused by ascorbic acid.

iv) Effect of growth factors

a) Epidermal growth factor

EGF inhibits alkaline phosphatase activity *in vitro* (Canalis, 1983a; Kumegawa *et al.*, 1983; Yokota *et al.*, 1986). EGF has also been shown to stimulate proliferation *in vitro* (Kumegawa *et al.*, 1983; Raisz *et al.*, 1980; Yokota *et al.*, 1986). In this study, EGF was shown to have no effect upon proliferation and to inhibit alkaline phosphatase activity, although by day 14 the latter effect was no longer significant statistically (Figure 8 and Table 14). *In vivo*, EGF receptors have not been found on functional osteoblasts but they are present on osteoblast-like cells and undifferentiated cells (Martineau-Doizé *et al.*, 1988). As it is the undifferentiated cells that possess a high proliferative potential, their absence or scarcity in the osteoblast cultures would explain the lack of any apparent mitogenic action. However, action of EGF on proliferation may have been influenced by that of growth factors in fetal calf serum used as a media supplement.

b) Platelet-derived growth factor

PDGF has been shown to inhibit alkaline phosphatase activity in osteoblasts derived from neonatal rat calvaria (Schmid *et al.*, 1984). The media used contained ascorbic acid, and in this study it was found that PDGF inhibited alkaline phosphatase activity in the presence of ascorbic acid, but was stimulatory in the absence of ascorbic acid (Table 15). This indicates an inhibitory effect of PDGF upon cells stimulated by ascorbic

acid to differentiate while itself providing a slight positive stimulus in relatively undifferentiated cells. As with EGF, no mitogenic action was found due to PDGF. In other studies, PDGF has has been shown to stimulate proliferation (Schmid *et al.*, 1984; Canalis, 1981; Canalis and Lian, 1988).

3. Osteogenesis *in vitro*

The formation of bone *in vitro* is dependent upon the synthesis and secretion of bone-related proteins, predominantly type I collagen, and the organised deposition of mineral crystals characterised by the nucleating action of matrix vesicles or collagen fibrils and the formation of a mineralisation front. *In vitro*, osteogenesis occurs in discrete nodules, each of which is probably derived from the differentiation and proliferation of one cellular unit (Bellows and Aubin, 1989; Bellows *et al.*, 1986; Bhargava *et al.*, 1988; Ecarot-Charrier *et al.*, 1983; Nefussi *et al.*, 1985).

i) Morphology

At confluence, the osteoblasts formed a layer of cuboidal cells (Figure 21) in a manner similar to that occurring in bone *in vivo* (Rodan and Rodan, 1984). After reaching confluence, the cultures formed multilayers of cells. Discrete patches of cells then developed into pronounced nodules (Figure 19a) which mineralised in the presence of ß-glycerophosphate (Figure 19b). Osteogenic nodules *in vitro* have also been seen in cultures of neonatal mouse calvarial cells established by migration (Ecarot-Charrier *et al.*, 1983) and in cultures of fetal rat calvarial cells isolated enzymatically (Bellows *et al.*, 1986; Bhargava *et al.*, 1988; Nefussi *et al.*, 1985). Cultures of bone marrow cells have also been shown to form osteogenic nodules (Maniatopoulos *et al.*, 1988).

Scanning EM showed the nodules to be raised above the main surface of the culture and to be heavily calcified (Figure 9). X-ray emission analysis of the nodules (Figure 10) showed the elemental composition to be mainly phosphorous and calcium, indicating the probable presence of hydroxyapatite (Osdoby and Caplan, 1980; Bhargava *et al.*, 1988; Maniatopoulos *et al.*, 1988).

Transmission EM showed the abundance of collagen in the extracellular space with the characteristic appearance of type I collagen: prominent transverse banding with a periodicity of approximately 62nm (Figure 14) (Piez, 1987). Type II collagen also presents periodic banding, but the fibrils are much thinner and the banding pattern is indistinct. Matrix vesicles were present amongst the collagen fibrils (Figure 13). *In vivo* matrix vesicles are closely associated with the onset of mineral deposition and may act as inducers of mineralisation (Anderson and Reynolds, 1973; Bonucci, 1987). These structures have previously been observed in areas of highly disorganised collagen fibres from cultures of bone cells from neonatal mice (Ecarot-Charrier *et al.*, 1988). However, compared to cartilage matrix vesicles, those of bone are small, scarce and rapidly calcified (Anderson, 1989). Consequently, they are not often seen.

Mineralisation of the extracellular matrix was confined to the osteogenic nodules and was found in association with collagen fibrils (Figure 14). The deposition of mineral was very clearly intimately connected with the fibrils as nascent mineral spicules were deposited along the fibrils perpendicular to the banding pattern. This type of mineralisation is similar to that found in lamellar bone. Few matrix vesicles and initial mineral deposition associated with the collagen fibrils (Gehron Robey *et al.*, 1988).

Morphologically the events occurring within the osteogenic nodules very closely resemble bone formation *in vivo*. The process of mineralisation appears to be regulated as mineral is only seen in discrete patches in association with collagen fibrils. The arrangement of the collagen in some areas was very organised and was probably controlled by osteoblastic activity (Jones and Boyde, 1976). The regulation of matrix synthesis and mineralisation is ultimately controlled by osteoblasts, which in turn differentiate *in vitro* into osteocytes and become embedded within the mineralised matrix (Figure 16). They were situated within lacunae and exhibited extended processes, which *in vivo* would probably penetrate into canaliculi (Marks and Popoff, 1985).

The cultures of neonatal mouse osteoblasts can therefore be considered to be osteogenic and to provide an *in vitro* model of bone formation.

ii) Effect of organic phosphate

ß-glycerophosphate has been shown to be necessary for the mineralisation of embryonic chick periostea *in vitro* (Tenenbaum, 1981). Quantification of the extent of mineralisation has shown a dose-dependent increase in calcium content with increasing ß-glycerophosphate concentrations in both neonatal mouse osteoblasts (Table 17) and a clonal mouse osteoblast cell line, MC3T3-E1 (Kodama *et al.*, 1986). The results of this study are therefore in complete agreement with the published work, validating the use of the colourimetric method.

iii) Effect of seeding density

The concept of the CFU-O implies an increased incidence of osteogenesis with increasing cell density. Cultures of fetal rat calvarial osteoblasts at low densities form calcified osteogenic nodules in direct proportion to the number of cells seeded (Bellows *et al.*, 1986; Bellows and Aubin, 1989). At higher densities, neonatal mouse osteoblasts produce a matrix which is increasingly calcified with increasing cell number at seeding (Table 18). However, at extremely high density, the amount of calcium deposited decreases, which may represent a general toxic effect due to accumulation of metabolic products, despite frequent media changes.

iv) Effect of hormones

a) Insulin

Insulin stimulates collagen synthesis in organ cultures of fetal rat calvaria (Canalis, 1983a). In cultures of neonatal mouse osteoblasts, insulin caused a significant increase in the extent of calcification (Figure 20), presumably this is secondary to the increased stimulation of collagen synthesis. In the absence of ascorbic acid, collagen synthesis is abnormal. However, under this condition, insulin at 5000ng/ml encouraged the formation of a calcified matrix, even though minimally.

b) Dexamethasone

Dexamethasone has been shown to cause a transient stimulation, followed by inhibition, of bone formation, alkaline phosphatase levels, proliferation and collagen synthesis in organ cultures (Canalis, 1983b; McCulloch and Tenenbaum, 1986). However, dexamethasone has also been shown to stimulate osteogenic nodule formation in cultures of isolated fetal rat osteoblasts (Bellows *et al.*, 1986; Bellows *et al.*, 1987). In this study, dexamethasone caused a significant decrease in the extent of calcification in cultures of neonatal mouse osteoblasts (Table 16).

These paradoxical effects are probably due to differences between species and the responsiveness of osteoprogenitors and more differentiated cells. At approximately the same plating density (3.0 to 4.0 x 10^4 cells/cm^2) as that used by Bellows and colleagues (1986) dexamethasone stimulated rat osteoblastic proliferation (Chen *et al.*, 1983). In cultures of rat cells, the stimulation of osteogenic nodule formation by dexamethasone is probably due to the increased cell number. The decrease in calcification (Table 16) may be due to a reduced volume of matrix following a highly significant decrease in cell number (Table 13) by day 14 of culture with dexamethasone.

v) Effect of growth factors

a) Epidermal growth factor

Short-term exposure (up to 4 days) to EGF causes a highly significant increase in nodule formation by rat calvarial cells (Antosz *et al.*, 1987). This may be due to the mitogenic effect of EGF, resulting in the formation of more nodule precursors without a concurrent committment to proliferation. Long-term exposure to EGF causes a highly significant decrease in the extent of calcification (Figure 21) and in the number of nodules (Antosz *et al.*, 1987). EGF has also been shown to selectively inhibit collagen synthesis (Canalis, 1983a; Canalis and Raisz, 1979; Hiramatsu *et al.*, 1982; Kumegawa *et al.*, 1983).

In organ cultures of fetal rat radii and ulnae, EGF causes a significant increase in resorption, as measured by the release of ^{45}Ca from pre-labeled bones (Raisz *et al.*, 1980). It would seem, therefore, that EGF could have a profound role in bone remodeling; stimulating resorption and inhibiting formation.

b) Platelet-derived growth factor

At 24 hours, PDGF has been shown to stimulate non-collagenous protein synthesis in organ cultures of fetal rat calvaria (Canalis, 1981; Canalis and Lian, 1988). At 60ng/ml (but not 100ng/ml) PDGF increased collagen synthesis, but by 96 hours the effect was inhibitory. The inhibition of matrix protein synthesis is probably the cause of the lack of calcification seen in cultures of neonatal mouse osteoblasts (pg 98).

4. Osteoblastic products *in vitro*

i) Haemopoietic cell growth factors

The importance of stromal elements during haemopoietic differentiation is indicated by the events occurring following marrow transplantation. Haemopoiesis by host cells is initiated by following osteogenic differentiation of the transplant (Friedenstein, 1976). Furthermore, the type of haemopoiesis is dependent upon the source of the donor marrow (Patt *et al.*, 1982). It has been known for some time that there is a non-haemopoietic compartment in the marrow cavity capable of producing colony stimulating factors (CSFs) (Chan and Metcalf, 1972). Marrow stromal cells lines have been shown to produce monocyte/macrophage colony stimulating factor (M-CSF) *in vitro* (Lanotte *et al.*, 1982). M-CSF acts upon granulocyte/macrophage colony forming cells (GM-CFC) *in vitro* resulting in the formation of monocyte/macrophage colonies. It has also been shown that glycosaminoglycans of the extracellular matrix of the marrow stroma can bind CSF and may act by;

i) locally concentrating tissue-specific growth factors,

ii) releasing active factors from this pool (Gordon *et al.*, 1987).

The stromal cells of bone have also been shown to produce haemopoietic growth factors. Primary cultures of neonatal mouse calvarial osteoblasts secrete granulocyte/macrophage CSF (GM-CSF), both constitutively (Figure 22) and following stimulation of monolayers by PTH and lipopolysaccharide (LPS) (Horowitz *et al.*, 1989). The increase in CSF production with time in culture (Figure 11) shows that the source is osteoblastic cells rather than contaminating marrow-derived cells. The osteoblastic origin of CSF has also been shown by the release of CSF from clonal osteoblastic cells lines, MC3T3-E1 and ROS, and neonatal mouse calvaria (Elford *et al.*, 1987; Felix *et al.*, 1988; Felix *et al.*, 1989; Shiina-Ishimi *et al.*, 1986; Weir *et al.*, 1989).

Media conditioned by MC3T3-E1 cells contained CSF and the synthesis of this factor increased with time in culture and was stimulated by interleukin 1, tumour necrosis factor α, LPS and the combined action of TGFß and EGF (Elford *et al.*, 1987; Felix *et al.*, 1989; Shiina-Ishimi *et al.*, 1986). ROS cells also secreted GM-CSF both constitutively and following stimulation by PTH and LPS (Weir *et al.*, 1989). The change with time of the type of colony formed suggests that osteoblasts may secrete both M-CSF and GM-CSF (Table 19).

Osteoblastic CSFs may play a role in the control of differentiation and proliferation of osteoclastic precursors. Co-culture of rat fetal bone rudiments with purified populations of G-CFC and GM-CFC resulted in a highly significant increase in the number of osteoclasts present within the rudiment. However, co-culture with M-CFC had no effect (Schneider and Relfson, 1988). It is possible, that osteoclasts and granulocytes share a common lineage and osteoclast formation could consequently be sensitive to the action of osteoblast CSFs. GM-CSF would encourage osteoclast formation whereas M-CSF would act in opposition, biasing GM-CFC differentiation towards monocyte/macrophage phenotypes, ie it would inhibit osteoclast formation. M-CSF has also been found to be a potent inhibitor of resorption by isolated osteoclasts incubated on bone slices (Hattersley *et al.*, 1988). In the same study, GM-CSF had no effect upon bone resorption.

Therefore, the stromal cells of bone, as well as of the marrow cavity, synthesise haemopoietic cell growth factors involved in the regulation of GM-CFC differentiation. Via this mechanism, it is likely that osteoblasts can contribute to the regulation of osteoclast formation.

ii) Effect on mesenchymal cells

Balb/c 3T3 cell lines are derived from cultures of minced Balb/c mouse embryos (Aaronson and Todaro, 1968). *In vitro*, these lines are capable of expressing a range of differentiated phenotypes and have been regarded as multipotent mesenchymal stem cells (Boone and Scott, 1980; Krawisz *et al.*, 1981; Sparks and Scott, 1986; Sparks *et al.*, 1986). Untransformed cells implanted *in vivo* as monolayers grown on plastic plates form undifferentiated sarcomas with differentiated foci of chondrocytes, fibroblasts and capillary pericytes (Boone and Scott, 1980). Suspensions of spontaneously transformed cells implanted sub-cutaneously form tumours exhibiting a range of morphologies, including myoblastic differentiation (Rubin *et al.*, 1985). The range of stromal phenotypes expressed by differentiated Balb/c 3T3 cell lines, *in vivo* and *in vitro*, raised the possibility of;

i) the potential of osteogenic differentiation, and
ii) a response to factor(s) produced by osteogenic cultures.

In the system used there was no discernible osteogenic differentiation of Balb/c 3T3 cells. The expression of alkaline phosphatase was minimal and in the presence of ß-glycerophosphate there was no deposition of mineral crystals (Table 19). Addition of concentrated osteoblast conditioned medium caused an inhibition of proliferation at low concentrations and a stimulation at high concentrations. The mitogenic activity may well be due to the presence of a bone cell-derived growth factor(s). However, without further investigations it is not possible to determine the cellular source or whether the activity is general or specific for mesenchymal cells.

The lack of osteogenic differentiation, both in the absence and presence of the osteoblast conditioned medium, may reflect inadequacies in the system employed. However, a negative result such as this can not make the distinction between faults inherent in the experiment and a genuine lack of osteogenic potential in the 3T3 cells. Indeed, if the osteogenic and chondrogenic lineages do share a common progenitor (Figure 2) then the expression of the chondrogenic phenotype may be an implicit indication of osteogenic potential.

However, it should be borne in mind that the pre-neoplasia and metaplasia exhibited by Balb/c 3T3 cell lines shows a lesser degree of regulation of differentiation and proliferation than that of normal cells. This is shown by the ability of 3T3 cells to first follow adipocytic differentiation and then still be capable of dedifferentiation and transdifferentiation (metaplasia) to the macrophage phenotype (Sparks *et al.*, 1986).

In summary, osteogenic differentiation can occur *in vitro* in cells derived from neonatal mouse calvaria. Quantification of parameters associated with the differentiated phenotype, alkaline phosphatase expression and calcium deposition, can provide information concerning the regulation and pattern of differentiation. Osteogenic cultures secrete factors into the media which stimulate differentiation of haemopoietic cells and the proliferation of mesenchymal cells. However, there is still a great deal to be understood concerning the interplay between bone cells, marrow stromal cells and haemopoietic cells. Although the existence, **in the adult mammal**, of a common stem cell is very unlikely there is a high degree of dependence between these systems in terms of cellular origins and control of differentiation and proliferation.

Future work on the differentiation of bone cells *in vitro* should concentrate on, not only the production of haemopoietic cell growth factors by osteoblasts, but also the role of marrow cells in stromal differentiation. If the stromal stem cell is located within the marrow cavity then the haemopoietic cells are ideally situated to exert control over differentiation and proliferation. *In vitro*, this can be studied using, for instance, long term

bone marrow culture conditioned medium and also co-culture of haemopoietic cells with osteoblasts and the mesenchymal stem cell line, Balb/c 3T3.

REFERENCES

REFERENCES

Aaronson, S.A. and Todaro, G.J. (1968) "Development of 3T3-like lines from Balb/c mouse embryo cultures: Transformation susceptibility to SV40" Journal of Cellular Physiology 72:141-148.

Anderson, H.C. (1989) "Mechanisms of mineral formation in bone" Laboratory Investigations 60:320-330.

Anderson, H.C. and Reynolds, J.J. (1973) "Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification" Developmental Biology 34:211-227.

Antosz, M.E., Bellows, C.G. and Aubin, J.E. (1987) "Biphasic effects of epidermal growth factor on bone nodule formation by isolated rat calvaria cells *in vitro*" Journal of Bone and Mineral Research 2:385-393.

Arnett, T.R. and Dempster, D.W. (1986) "Effect of pH on bone resorption by rat osteoclasts *in vitro*" Endocrinology 119:119-124.

Arsenault, A.L. and Ottensmeyer, F.P. (1984) "Visualisation of early intramembranous ossification by electron microscopic and spectroscopic imaging" The Journal of Cell Biology 98:911-921.

Ashton, B.A., Abdullah, F., Cave, J., Williamson, M., Sykes, B.C., Couch, M. and Poser, J.W. (1985) "Characterization of cells with high alkaline phosphatase activity derived from human bone and marrow: Preliminary assessment of their osteogenicity" Bone 6:313-319.

Ashton, B.A., Allen, T.D., Howlett, C.R., Eaglesom, C.C., Hattori, A. and Owen, M. (1980) "Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*" Clinical Orthopaedics and Related Research 151:294-307.

Ashton, B.A., Couch, M. and Owen, M. (1986) "The influence of ß—glycerophosphate on human bone derived cells" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y., ed.) pp 349-352. Elsevier Science Publishers.

Ashton, B.A., Eaglesom, C.C., Bab, I. and Owen, M.E. (1984) "Distribution of fibroblastic colony-forming cells in rabbit bone marrow and assay of their osteogenic potential by an *in vivo* diffusion chamber method" Calcified Tissue International 36:83-86.

Bab, I., Ashton, B.A., Syftestad, G.T. and Owen, M.E. (1984) "Assessment of an *in vivo* diffusion chamber method as a quantitative assay for osteogenesis" Calcified Tissue International 36:77-82.

Bellows, C.G. and Aubin, J.E. (1989) "Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells *in vitro*" Developmental Biology 133:8-13.

Bellows, C.G., Aubin, J.E. and Heersche, J.N.M. (1987) "Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*" Endocrinology 121:1985-1992.

Bellows, C.G., Aubin, J.E., Heersche, J.N.M. and Antosz, M.E. (1986) "Mineralized bone nodules formed *in vitro* from enzymatically released rat calvarial cell populations" Calcified Tissue International 38:143-154.

Beresford, J.N. (1989) "Osteogenic stem cells and the stromal system of bone and marrow" Clinical Orthopaedics and Related Research 240:270-280.

de Bernard, B., Bianco, P., Bonucci, E., Costantini, M., Lunazzi, G.C., Martinuzzi, P., Modricky, C., Moro, L., Panfili, E., Pollesello, P., Stagni, N. and Vittur, F. (1986) "Biochemical and immunohistochemical evidence that in cartilage an alkaline phosphatase is a Ca^{2+}-binding glycoprotein" The Journal of Cell Biology 103:1615-1623.

Bernick, S., Paule, W., Ertl, D., Nishimoto, S.K. and Nimni, M.E. (1989) "Cellular events associated with the induction of bone by demineralized bone" Journal of Orthopaedic Research 7:1-11.

Bhargava, U., Bar-Lev, M., Bellows, C.G. and Aubin, J.E. (1988) "Ultrastructural analysis of bone nodules formed *in vitro* by isolated fetal rat calvaria cells" Bone 9:155-163.

Binderman, I., Duskin, D., Harell, A., Katzir (Katchalski), E. and Sachs, L. (1974) "Formation of bone tissue in culture from isolated bone cells" Journal of Cell Biology 61:427-439.

Blair, H.C., Kahn, A.J., Crouch, E.C., Jeffrey, J.J. and Teitelbaum, S.L. (1986) "Isolated osteoclasts resorb the organic and inorganic components of bone" The Journal of Cell Biology 102:1164-1172.

Bonucci, E. (1981) "New knowledge on the origin, function and fate of osteoclasts" Clinical Orthopaedics and Related Research 158:252-269.

Bonucci, E. (1987) "Is there a calcification factor common to all calcifying matrices?" Scanning Microscopy 1:1089-1102.

Boone, C.W. and Scott, R.E. (1980) "Plate-induced tumours of Balb/3T3 cells exhibiting foci of differentiation into pericytes, chondrocytes, and fibroblasts" Journal of Supramolecular Structure 14:233-240.

Boonekamp, P.M., Hekkelman, J.W., Jilka, R.L. and Cohn, D.V. (1981) "An improved method for isolating hormonally responsive cells from mouse calvaria" Calcified Tissue International 33:291.

Brown, B.L., Albano, J.D.M., Ekins, R.P., Sgherzi, A.M. and Tampion, W. (1971) "A simple and sensitive saturation assay for the measurement of adenosine 3':5'-cyclic monophosphate" Biochemistry Journal 121:561-562.

Bushinsky, D.A., Chabala, J.M. and Levi-Setti, R. (1989) "Ion microprobe analysis of mouse calvariae *in vitro*: Evidence for a "bone membrane"" American Journal of Physiology 256:E152-E158.

Butler, W.T. (1987) "Mineralised tissues: An overview" Methods in Enzymology 145:255-261.

Canalis, E. (1981) "Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria" Metabolism 30:970-975.

Canalis, E. (1983a) "Effects of hormones and growth factors on alkaline phosphatase activity and collagen synthesis in cultures rat calvariae" Metabolism 32:14-20.

Canalis, E. (1983b) "Effect of glucocorticoids on type I collagen synthesis, alkaline phosphatase activity, and deoxyribonucleic acid content in cultured rat calvariae" Endocrinology 112:931-939.

Canalis, E. (1987) "Effects of tumour necrosis factor on bone formation *in vitro*" Endocrinology 121:1596-1604.

Canalis, E. (1988) "Bone-related growth factors" Triangle 27:11-19.

Canalis, E. and Centrella, M. (1986) "Isolation of a nontransforming bone-derived growth factor from medium conditioned by fetal rat calvariae" Endocrinology 118:2002-2008.

Canalis, E., Centrella, M. and McCarthy, T. (1988a) "Effects of basic fibroblast growth factor on bone formation *in vitro*" Journal of Clinical Investigation 81:1572-1577.

Canalis, E. and Lian, J.B. (1988) "Effects of bone associated growth factors on DNA, collagen and osteocalcin synthesis in cultured fetal rat calvariae" Bone 9:243-246.

Canalis, E., McCarthy, T. and Centrella, M. (1988b) "Growth factors and the regulation of bone remodelling" Journal of Clinical Investigation 81:277-281.

Canalis, E., McCarthy, T. and Centrella, M. (1988c) "Isolation and characterization of insulin-like growth factor I (somatomedin-C) from cultures of fetal rat calvariae" Endocrinology 122:22-27.

Canalis, E. and Raisz, L.G. (1979) "Effect of epidermal growth factor on bone formation *in vitro*" Endocrinology 104:862—869.

Caplan, A.I. (1987) "Bone development and repair" BioEssays 6:171–175.

Caplan, A.I. and Pechak, D.G. (1987) "The cellular and molecular embryology of bone formation" in "Bone and Mineral Research/5" (Peck, W.A., ed.) pp 117-183. Elsevier Science Publishers.

Carrington, J.L., Roberts, A.B., Flanders, K.C., Roche, N.S. and Reddi, A.H. (1988) "Accumulation, localization, and compartmentation of transforming growth factor ß during endochondral bone development" The Journal of Cell Biology 107:1969-1975.

Centrella, M. and Canalis, E. (1985) "Transforming and nontransforming growth factors are present in medium conditioned by fetal rat calvariae" Proceedings of the National Academy of Sciences 82:7335-7339.

Centrella, M., McCarthy, T.L. and Canalis, E. (1987a) "Mitogenesis in fetal rat bone cells simultaneously exposed to type ß transforming growth factor and other growth regulators" Federation of American Societies for Experimental Biology Journal 1:312-317.

Centrella, M., McCarthy, T.L. and Canalis, E. (1987b) "Transforming growth factor ß is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone" The Journal of Biological Chemistry 252:2869-2874.

Centrella, M., McCarthy, T.L. and Canalis, E. (1988) "Parathyroid hormone modulates transforming growth factor ß activity and binding in osteoblast-enriched cell cultures from fetal rat parietal bone" Proceedings of the National Academy of Sciences 85:5889-5893.

Chambers, T.J. (1980) "The cellular basis of bone resorption" Clinical Orthopaedics and Related Research 151:283-293.

Chambers, T.J. and Ali, N.N. (1983) "Inhibition of osteoclastic motility by prostaglandins I_2, E_1, E_2 and 6-oxo-E_1" Journal of Pathology 139:383-397.

Chambers, T.J., Athanasou, N.A. and Fuller, K. (1984) "Effect of parathyroid hormone and calcitonin on the cytoplasmic spreading of isolated osteoclasts" Journal of Endocrinology 102:281-286.

Chambers, T.J. and Fuller, K. (1985) "Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact" Journal of Cell Science 76:155-165.

Chambers, T.J. and Magnus, C.J. (1982) "Calcitonin alters behaviour of isolated osteoclasts" Journal of Pathology 136:27-39.

Chambers, T.J. and Moore, A. (1983) "The sensitivity of isolated osteoclasts to morphological transformation by calcitonin" Journal of Clinical and Endocrinological Metabolism 57:819-824.

Chan, S.H. and Metcalf, D. (1972) "Local production of colony-stimulating factor within the bone marrow: Rôle of nonhematopoietic cells" Blood 40:646-653.

Chen, T.L., Cone, C.M. and Feldman, D. (1983) "Glucocorticoid modulation of cell proliferation in cultured osteoblast-like cells: Differences between rat and mouse" Endocrinology 112:1739-1745.

Chen, T.L. and Feldman, D. (1979) "Glucocorticoid receptors and actions in subpopulations of cultured rat bone cells" Journal of Clinical Investigation 63:750-758.

Chyun, Y.S., Kream, B.E. and Raisz, L.G. (1984) "Cortisol decreases bone formation by inhibiting periosteal cell proliferation" Endocrinology 114:477-480.

de Clerck, Y.A. and Jones, P.A. (1980) "The effect of ascorbic acid on the nature and production of collagen and elastin by rat smooth-muscle cells" Biochemistry Journal 186:217-225.

Clezardin, P., Malaval, L., Ehrensperger, A.-S., Delmas, P.D., Dechavanne, M. and McGregor, J.L. (1988) "Complex formation of human thrombospondin with osteonectin" European Journal of Biochemistry 175:275-284.

Dickson, I. (1987) "New approaches to vitamin D" Nature 325:18.

Dietrich, J.W., Canalis, E.M., Maina, D.M. and Raisz, L.G. (1976) "Hormonal control of bone collagen synthesis *in vitro*: Effects of parathyroid hormone and calcitonin" Endocrinology 98:943-949.

Doi, Y., Okuda, R., Takezawa, Y., Shibata, S., Moriwaki, Y., Wakamatsu, N., Shimizu, N., Moriyama, K. and Shimokawa, H. (1989) "Osteonectin inhibiting *de novo* formation of apatite in the presence of collagen" Calcified Tissue International 44:200-208.

Dziedzic-Goclawska, A., Emerich, J., Grzesik, W., Stachowicz, Michalik, J. and Ostrowski, K. (1988) "Differences in the kinetics of the mineralization process in endochondral and intramembranous osteogenesis in human fetal development" Journal of Bone and Mineral Research 3:533-539.

Ecarot-Charrier, B. and Broekhuyse, H. (1987) "Proteoglycans synthesized by cultured mouse osteoblasts" The Journal of Biological Chemistry 262:5345-5351.

Ecarot-Charrier, B., Glorieux, F.H., van der Rest, M. and Pereira, G. (1983) "Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture" The Journal of Cell Biology 96:639-643.

Ecarot-Charrier, B., Shepard, N., Charette, G., Grynpas, M. and Glorieux, F.H. (1988) "Mineralization in osteoblast cultures: A light and electron microscopic study" Bone 9:147-154.

Elford, P.R., Felix, R., Cecchini, M., Trechsel, U. and Fleisch, H. (1987a) "Murine osteoblastic cells and the osteogenic cell MC3T3-E1 release a macrophage colony-stimulating activity in culture" Calcified Tissue International 41:151–156.

Elford, P.R., Guenther, H.L., Felix, R., Cecchini, M.G. and Fleisch, H. (1987b) "Transforming growth factor-ß reduces the phenotypic expression of osteoblastic MC3T3-E1 cells in monolayer culture" Bone 8:259-262.

Ellingsworth, L.R., Brennan, J.E., Fok, K., Rosen, D.M., Bentz, H., Piez, K.A. and Seyedin, S.M. (1986) "Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor ß" The Journal of Biological Chemistry 261:12362-12367.

Endo, H. (1960) "Ossification in tissue culture" Experimental Cell Research 21:151-163.

Ernst, M. and Froesch, E.R. (1988) "Growth hormone dependent stimulation of osteoblast-like cells in serum-free culture via local synthesis of insulin-like growth factor I" Biochemical and Biophysical Research Communications 151:142-147.

Farley, J.R. and Baylink, D.J. (1982) "Purification of a skeletal growth factor from human bone" Biochemistry 21:3502-3507.

Farley, J.R., Tarbaux, N., Murphy, L.A., Masuda, T. and Baylink, D.J. (1987) "*In vitro* evidence that bone formation may be coupled to resorption by release of mitogen(s) from resorbing bone" Metabolism 36:314-321.

Felix, R., Elford, P.R., Stoercklé, C., Cecchini, M., Wetterwald, A., Trechsel, U., Fleisch, H. and Stadler, B.M. (1988) "Production of hemopoietic growth factors by bone tissue and bone cells in culture" Journal of Bone and Mineral Research 3:27-36.

Felix, R., Fleisch, H. and Elford, P.R. (1989) "Bone-resorbing cytokines enhance release of macrophage colony-stimulating activity by the osteoblastic cell MC3T3-E1" Calcified Tissue International 44:356-360.

Fisher, L.W., Termine, J.D. and Young, M.F. (1989) "Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species" The Journal of Biological Chemistry 264:4571-4576.

Friedenstein, A.J. (1976) "Precursor cells of mechanocytes" International Review of Cytology 47:327-355.

Friedenstein, A.J., Chailakhjan, R.K. and Gerasimov, U.V. (1987) "Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers" Cell and Tissue Kinetics 20:263-272.

Friedenstein, A.J., Chailakhjan, R.K. and Lalykina, K.S. (1970) "The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells" Cell and Tissue Kinetics 3:393-403.

Friedenstein, A.J. and Kuralesova, A.I. (1971) "Osteogenic precursor cells of bone marrow in radiation chimeras" Transplantation 12:99-108.

Gallagher, J.A., Woods, D.A., Beresford, J.N., Sheard, C.E., Dillon, J.P. and Russell, R.G.G. (1986) "Evaluation of osteocalcin and other bone matrix proteins as markers of the osteoblastic phenotype *in vitro*" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y. ed.) pp 119–127. Elsevier Science Publishers.

Gehron Robey, P., Fisher, L.W., Young, M.F. and Termine, J.D. (1988) "The biochemistry of bone" in "Osteoporosis: Etiology, Diagnosis, and Management" (Riggs, B.L. and Melton, L.J., eds.) pp 95-109. Raven Press, New York.

Gehron Robey, P., Young, M.F., Fisher, L.W. and McClain, T.D. (1989) "Thrombospondin is an osteoblast-derived component of mineralised extracellular matrix" The Journal of Cell Biology 108:719-727.

Gehron Robey, P., Young, M.F., Flanders, K.C., Roche, N.S., Kondaiah, P., Reddi, A.H., Termine, J.D., Sporn, M.B. and Roberts, A.B. (1987) "Osteoblasts synthesize and respond to transforming growth factor-type ß (TGF-ß) *in vitro*" The Journal of Cell Biology 105:457-463.

Gendreau, M.A., Krishnaswamy, S. and Mann, K.G. (1989) "The interaction of bone Gla protein (osteocalcin) with phospholipid vesicles" The Journal of Biological Chemistry 264:6972-6978.

Giraud-Guille, M.M. (1988) "Twisted plywood architecture of collagen fibrils in human compact bone osteons" Calcified Tissue International 42:167-180.

Globus, R.K., Patterson-Buckendahl, P. and Gospodarowicz, D. (1988) "Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factorß" Endocrinology 123:98-105.

Globus, R.K., Plouet, J. and Gospodarowicz, D. (1989) "Cultured bovine bone cells synthesise basic fibroblast growth factor and store it in their extracellular matrix" Endocrinology 124:1539-1547.

Goldberg, H.A., Domenicucci, C., Pringle, G.A. and Sodek, J. (1988) "Mineral-binding proteoglycans of fetal porcine calvarial bone" The Journal of Biological Chemistry 263:12092-12101.

Gordon, M.Y., Riley, G.P., Watt, S.M., Greaves, M.F. (1987) "Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment" Nature 326:403-405.

Gray, T.K., Mohan, S., Linkhart, T.A. and Baylink, D.J. (1989) "Estradiol stimulates *in vitro* the secretion of insulin-like growth factors by the clonal osteoblastic cell line, UMR106" Biochemical and Biophysical Research Communications 158:407-412.

Green, E., Hinton, C. and Triffitt, J.T. (1986) "The effect of decalcified bone matrix on the osteogenic potential of bone marrow" Clinical Orthopaedics and Related Research 205:292-298.

Groot, C.G., Danes, J.K., Blok, J., Hoogendijk, A. and Hauschka, P.V. (1986) "Light and electron demonstration of osteocalcin antigenicity in embryonic and adult rat bone" Bone 7:379-385.

Groot, C.G., Moskalewski, S., Scherft, J.P. and Boonekamp, P.M. (1983) "Electron microscopy of bone formed by syngeneic transplanted calvarial osteoblasts" Cell Biology International Reports 7:577.

Guenther, H.L., Cecchini, M.G., Elford, P.R. and Fleisch, H. (1988) "Effects of transforming growth factor type beta upon bone cell populations grown either in monolayer or semisolid medium" Journal of Bone and Mineral Research 3:269-278.

Gutierrez, G.E., Mundy, G.R., Derynck, R., Hewlett, E.L. and Katz, M.S. (1987) "Inhibition of parathyroid hormone-responsive adenylate cyclase in clonal osteoblast-like cells by transforming growth factor a and epidermal growth factor" The Journal of Biological Chemistry 262:15845-15850.

Habuchi, H., Conrad, H.E. and Glaser, J.H. (1985) "Coordinate regulation of collagen and alkaline phosphatase levels in chick embryo chondrocytes" The Journal of Biological Chemistry 260:13029-13034.

Hahn, T.J., Westbrook, S.L. and Halstead, L.R. (1984) "Cortisol modulation of osteoblast metabolic activity in cultured neonatal rat bone" Endocrinology 114:1864-1870.

Hall, B.K. (1975) "The origin and fate of osteoclasts" The Anatomical Record 183:1-12.

Hall, B.K. (1988) "The embryonic development of bone" American Scientist 76:174-181.

Hall, G.E. and Kenny, A.D. (1986) "Bone resorption induced by parathyroid hormone and dibutyryl cyclic AMP: Role of carbonic anhydrase" The Journal of Pharmacology and Experimental Therapeutics 238:778-782.

Hanaoka, H., Yabe, H. and Bun, H. (1989) "The origin of the osteoclast" Clinical Orthopaedics and Related Research 139:286-298.

Harada, K., Oida, S. and Sasaki, S. (1988) "Chondrogenesis and osteogenesis of bone marrow-derived cells by bone-inductive protein" Bone 9:177-183.

Harakas, N.K. (1984) "Demineralized bone-matrix-induced osteogenesis" Clinical Orthopaedics and Related Research 188:239-251.

Hattersley, G., Dorey, E., Horton, M.A. and Chambers, T.J. (1988) "Human macrophage colony-stimulating factor inhibits resorption by osteoclasts disaggregated from rat bone" Journal of Cellular Physiology 137:199-203.

Hauschka, P.V., Mavrakos, A.E., Iafrati, M.D., Doleman, S.E. and Klagsbrun, M. (1986) "Growth factors in bone matrix" The Journal of Biological Chemistry 261:12665-12674.

Hauschka, P.V. and Reddi, A.H. (1980) "Correlation of the appearance of Á-carboxyglutamic acid with the onset of mineralisation in developing endochondral bone" Biochemical and Biophysical Research Communications 92:1037-1041.

Heath, J.K., Atkinson, S.J., Meikle, M.C. and reynolds, J.J. (1984) "Mouse osteoblasts synthesise collagenase in response to bone resorbing agents" Biochimica et Biophysica Acta 802:151-154.

Heersche, J.N.M. (1978) "Mechanisms of osteoclastic bone resorption: A new hypothesis" Calcified Tissue Research 26:81-84.

Hekkelman, J.W. and Moskalewski, S. (1969) "The effect of parathyroid extract on the alkaline phosphatase activity of the surface of isolated bone cells" Experimental Cell Research 58:283-288.

Hickman, J. and McElduff, A. (1989) "Insulin promotes growth of the cultured rat osteosarcoma cell line UMR-106-01: An osteoblast-like cell" endocrinology 124:701-706.

Hiramatsu, M., Kumegawa, M., Hatakeyama, K., Yajima, T., Minami, N. and Kodama, H. (1982) "Effect of epidermal growth factor on collagen synthesis in osteoblastic cells derived from newborn mouse calvaria" Endocrinology 111:1810-1716.

Hock, J.M., Centrella, M. and Canalis, E. (1988) "Insulin-like growth factor I has independent effects on bone matrix formation and cell replication" Endocrinology 122:254-260.

Horowitz, M.C., Coleman, D.L., Flood, P.M., Kupper, T.S. and Jilka, R.L. (1989) "Parathyroid hormone and lipopolysaccharide induce murine osteoblast-like cells to secrete a cytokine indistinguishable from granulocyte-macrophage colony-stimulating factor" Journal of Clinical Investigation 83:149-157.

Ibbotson, K.J., Harrod, J., Gowen, M., D'Souza, S., Smith, D.D., Winkler, M.E., Derynck, R. and Mundy, G.R. (1986) "Human recombinant transforming growth factor a stimulates bone resorption and inhibits formation *in vitro*" Proceedings of the National Academy of Sciences 83:2228-2232.

Ituarte, E.A., Ituarte, H.G. and Hahn, T.J. (1988) "Insulin and glucose regulation of glycogen synthesase in rat calvarial osteoblastlike cells" Calcified Tissue International 42:351-357.

Jones, S.J. and Boyde, A. (1976) "Is there a relationship between osteoblasts and collagen orientation in bone?" Israeli Journal of Medical Science 12:98-107.

Jones, S.J. and Boyde, A. (1976) "Morphological changes of osteoblasts *in vitro*" Cell and Tissue Research 166:101–107.

Jones, S.J. and Boyde, A. (1977) "The migration of osteoblasts" Cell and Tissue Research 184:179-193.

Jotereau, F.V. and Le Douarin, N.M. (1978) "The developmental relationship between osteocytes and osteoclasts: A study using the quail-chick nuclear marker in endochondral ossification" Developmental Biology 63:253-265.

Jundt, G., Berghäuser, K.-H., Termine, J.D. and Schulz, A. (1987) "Osteonectin - a differentiation marker of bone cells" Cell and Tissue Research 248:409-415.

Kahn, A.J., Fallon, M.D. and Teitelbaum, S.L. (1983) "Structure-function relationships in bone: An examination of events at the cellular level" in "Bone and Mineral Research/2" (Peck, W.A., ed.) pp 125-174. Elsevier Science Publishers.

Kahn, A.J. and Simmons, D.J. (1975) "Investigation of cell lineage in bone using a chimera of chick and quail embryonic tissue" Nature 258:325-327.

Kinne, R.W. and Fisher, L.W. (1987) "Keratan sulfate proteoglycan in rabbit compact bone is bone sialoprotein II" The Journal of Biological Chemistry 262:10206-10211.

Ko, J.S. and Bernard, G.W. (1981) "Osteoclast formation *in vitro* from bone marrow mononuclear cells in osteoclast-free bone" American Journal of Anatomy 161:415-425.

Koch, C.J. and Biaglow, J.E. (1978) "Toxicity, radiation sensitivity modification, and metabolic effects of dehydroascorbate and ascorbate" Journal of Cellular Physiology 94:299-306.

Kodama, H., Amagai, Y., Sudo, H., Kasai, S. and Yamamoto, S. (1981) "Establishment of a clonal osteogenic cell line from newborn mouse calvaria" Japanese Journal of Oral Biology 23:899-901.

Kodama, H., Amagai, Y., Sudo, H., Ohno, T. and Iijima, K.-I. (1986) "Culture conditions affecting differentiation and calcification in the MC3T3-E1 osteogenic cell line" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y., ed.) pp 297-302. Elsevier Science Publishers.

Krawisz, B.R., Florine, D.L. and Scott, R.E. (1981) "Differentiation of fibroblast-like cells into macrophages" Cancer Research 41:2891-2899.

Kumegawa, M., Hiramatsu, M., Hatakeyama, K., Yajima, T., Kodama, H., Osaki, T. and Kurisu, K. (1983) "Effects of epidermal growth factor on osteoblastic cells *in vitro*" Calcified Tissue International 35:542-548.

Lanotte, M., Metcalf, D. and Dexter, T.M. (1982) "Production of monocyte/macrophage colony-stimulating factor by preadipocyte cell lines derived from murine marrow stroma" Journal of Cellular Physiology 112:123-127.

Lau. K.-H.W., Jennings, J.C.and Baylink, D.J. (1988) "Bovine skeletal growth factor stimulates protein phosphorylation of chicken bone cells *in vitro*" International Journal of Biochemistry 20:1443-1450.

Laurent, G.J. (1987) "Dynamic state of collagen: Pathways of collagen degradation *in vivo* and their possible role in regulation of collagen mass" American Journal of Physiology 252:C1-C9.

Levy, J.R., Murray, E., Manolagas, S. and Olefsky, J.M. (1986) "Demonstration of insulin receptors and modulation of alkaline phosphatase activity by insulin in rat osteoblastic cells" Endocrinology 119:1786-1792.

Lian, J.B., Coutts, M. and Canalis, E. (1985) "Studies of hormonal regulation of osteocalcin synthesis in cultured rat calvariae" The Journal of Biological Chemistry 260:8706—8710.

Linkhart, S., Mohan, S., Linkhart, T.A., Kumegawa, M. and Baylink, D.J. (1986) "Human skeletal growth factor stimulates collagen synthesis and inhibits proliferation in a clonal osteoblast cell line (MC3T3-E1)" Journal of Cellular Physiology 128:307-312.

Lowry, O.H. (1955) "Micromethods for the assay of enzymes - alkaline phosphatase" Methods in Enzymology 4:371.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with the folin phenol reagent" Journal of Biological Chemistry 193:265-275.

Luben, R.A., Wong, G.L. and Cohn, D.V. (1976) "Biochemical characterization with parathormone and calcitonin: Provisional identification of osteoclasts and osteoblasts" Endocrinology 99:526-534.

Lucas, P.A., Price, P.A. and Caplan, A.I. (1988) "Chemotactic response of mesenchymal cells, fibroblasts and osteoblast-like cells to bone Gla protein" Bone 9:319-323.

Luria, E.A., Kuznetsov, S.A., Genkina, E.N. and Fridenshtein, A.Y. (1988) "Bone tissue formation by mouse bone marrow cell suspension in organ cultures" Bulletin of Experimental Biology and Medicine 105:856-859.

Luria, E.A., Owen, M.E., Friedenstein, A.J., Morris, J.F. and Kuznetsow, S.A. (1987) "Bone formation in organ cultures of bone marrow" Cell and Tissue Research 248:449-454.

Luriya, E.A., Owen, M., Fridenshtein, A.Y., Kuznetsov, S.A., Genkina, E.N. and Gosteva, V.V. (1986) "Bone formation in bone marrow organ cultures" Bulletin of Experimental Biology and Medicine 101:520-525.

MacDonald, B.R., Gallagher, J.A., Ahnfelt-Ronne, I., Beresford, J.N., Gowen, M. and Russell, R.G.G. (1984) "Effects of bovine parathyroid hormone and 1,25-dihydroxyvitamin D_3 on the production of prostaglandins by cells derived from human bone" Federation of European Biochemical Societies Letters 169:49-52.

Maclean, N. and Hall, B.K. (1987) "Cell commitment and differentiation" Cambridge University Press.

Mahy, P.R. and Urist, M.R. (1988) "Experimental heterotopic bone formation induced by bone morphogenetic protein and recombinant human interleukin-1B" Clinical Orthopaedics and Related Research 237:236-244.

Majeska, R.J., Rodan, S.B. and Rodan, G.A. (1978) "Maintenance of parathyroid hormone response in clonal rat osteosarcoma lines" Experimental Cell Research 111:465-468.

Malone, J.D., Teitelbaum, S.L., Griffin, G.L., Senior, R.M. and Kahn, A.J. (1982) "Recruitment of osteoclast precursors by purified bone matrix constituents" The Journal of Cell Biology 92:227-230.

Maniatopoulos, C., Sodek, J. and Melcher, A.H. (1988) "Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats" Cell and Tissue Research 254:317-330.

Mann, S. (1988) "Molecular recognition in biomineralization" Nature 332:119-124.

Marcus, R. (1987) "Normal and abnormal bone remodeling in man" Annual Review of Medicine 38:129-141.

Mardon, H.J., Bee, J., von der Mark, K. and Owen, M.E. (1987) "Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats" Cell and Tissue Research 250:157-165.

Marks, S.C. and Popoff, S.N. (1988) "Bone cell biology: The regulation of development, structure, and function in the skeleton" The American Journal of Anatomy 183:1-44.

Martin, T.J., Ng, K.W. and Nicholson, G.C. (1988) "Cell biology of bone" Baillière's Clinical Endocrinology and Metabolism 2:1-29.

Martineau-Doizé, B., Lai, W.H., Warshawsky, H. and Bergeron, J.J.M. (1988) "*In vivo* demonstration of cell types in bone that harbor epidermal growth factor receptors" Endocrinology 123:841-858.

Marvaso, V. and Bernard, G.W. (1977) "Initial intramembranous osteogenesis *in vitro*" American Journal of Anatomy 149:453-467.

McCarthy, T.L., Centrella, M. and Canalis, E. (1989a) "Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures" Endocrinology 124:301-309.

McCarthy, T.L., Centrella, M. and Canalis, E. (1989b) "Parathyroid hormone enhances the transcription and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone" Endocrinology 124:1247-1253.

McCulloch, C.A.G and Tenenbaum, H.C. (1986) "Dexamethasone induces proliferation and terminal differentiation of osteogenic cells in tissue culture" The Anatomical Record 215:397-402.

McCulloch, C.A.G., Tenenbaum, H.C., Fair, C.A. and Birek, C. (1989) "Site-specific regulation of osteogenesis: Maintenance of discrete levels of phenotypic expression *in vitro*" The Anatomical Record 223:27-34.

McLean, F.M., Keller, P.J., Genge, B.R., Walters, S.A. and Wuthier, R.E. (1987) "Disposition of preformed mineral in matrix vesicles" The Journal of Biological Chemistry 262:10481—10488.

McSheehy, P.M.J. and Chambers, T.J. (1986a) "Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone" Endocrinology 118:824-828.

McSheehy, P.M.J. and Chambers, T.J. (1986b) "Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption" Endocrinology 119:1654-1659.

Metcalf, D., Bradley, T.R. and Robinson, W. (1966) "Analysis of colonies developing *in vitro* from mouse bone marrow cells stimulated by kidney feeder layers or leukemic serum" Journal of Cellular Physiology 69:93-108.

Mikhailova, L.N. and Pal'tsyn, A.A. (1986) "Osteogenic precursor cells in reparative osteogenesis" Bulletin of Experimental Biology and Medicine 101:854-858.

Miller, S.C., Bowman, B.M., Smith, J.M. and Jee, W.S.S. (1980) "Characterization of endosteal bone-lining cells from fatty marrow bone sites in adult beagles" The Anatomical Record 198:163-173.

Miller, S.C. and Jee, W.S.S. (1987) "The bone lining cell: A distinct phenotype?" Calcified Tissue International 41:1–5.

Moskalewski, S., Boonekamp, P.M. and Scherft, J.P. (1983) "Bone formation by isolated calvarial osteoblasts in syngeneic and allogeneic transplants: Light microscopic observations" The American Journal of Anatomy 167:249-263.

Mundy, G.R. and Poser, J.W. (1983) "Chemotactic activity of the γ-carboxyglutamic acid containing protein in bone" Calcified Tissue International 35:164-168.

Murad, S., Grove, D., Lindberg, K.A., Reynolds, G.G., Sivarajah, A. and Pinnell, S.R. (1981) "Regulation of collagen synthesis by ascorbic acid" Proceedings of the National Academy of Sciences 78:2879-2882.

Muthukumaran, N. and Reddi, A.H. (1985) "Bone matrix-induced local bone induction" Clinical Orthopaedics and Related Research 200:159-164.

Nefussi, J.-P., Boy-Lefevre, M.L., Boulekbache, H. and Forest, N. (1985) "Mineralization *in vitro* of matrix formed by osteoblasts isolated by collagenase digestion" Differentiation 29:160-168.

Nicholson, G.C., Moseley, J.M., Sexton, P.M., Mendelsohn, F.A.O. and Martin, T.J. (1986) "Abundant calcitonin receptors in isolated rat osteoclasts" Journal of Clinical Investigation 78:355-360.

Nijweide, P.J. and Mulder, R.J.P. (1986) "Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes" Histochemistry 84:342-347.

Nijweide, P.J., van der Plas, A. and Scherft, J.P. (1981) "Biochemical and histological studies on various bone cell preparations" Calcified Tissue International 33:529-540.

Noda, M. (1989) "Transcriptional regulation of osteocalcin production by transforming growth factor-ß in rat osteoblast-like cells" Endocrinology 124:612-617.

Noda, M. and Rodan, G.A. (1986) "Type-ß transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells" Biochemical and Biophysical Research Communications 140:56-65.

Noda, M. and Rodan, G.A. (1987) "Type ß transforming growth factor (TGFß) regulation of alkaline phosphatase expression and other phenotypically-related mRNAs in osteoblastic rat osteosarcoma cells" Journal of Cellular Physiology 133:426-437.

Oldberg, Å., Franzén, A. and Heinegård, D. (1986) "Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an arg-gly-asp cell-binding sequence" Proceedings of the National Academy of Sciences 83:8819–8823.

Oldberg, Å., Franzén, A. and Heinegård, D. (1988a) "The primary structure of a cell-binding bone sialoprotein" The Journal of Biological Chemistry 263:19430-19432.

Oldberg, Å., Franzén, A., Heinegård, D., Pierschbacher, M. and Ruoslahti, E. (1988b) "Identification of a bone sialoprotein receptor in osteosarcoma cells" The Journal of Biological Chemistry 263:19433-19436.

Ornoy, A., Goodwin, D., Noff, D. and Edelstein, S. (1978) "24,25-dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation" Nature 276:517-519.

Osdoby, P. and Caplan, A.I. (1979) "Osteogenesis in cultures of limb mesenchymal cells" Developmental Biology 73:84-102.

Osdoby, P. and Caplan, A.I. (1980) "A scanning electron microscopic investigation of *in vitro* osteogenesis" Calcified Tissue International 30:43-50.

Osdoby, P. Krukowski, M., Oursler, M.J. and Salino-Hugg, T. (1987) "The origin, development and regulation of osteoclasts" BioEssays 7:30-34.

Otsuka, K., Yao, K.-L., Wasi, S., Tung, P.S., Aubin, J.E., Sodek, J. and Termine, J.D. (1984) "Biosynthesis of osteonectin by fetal porcine calvarial cells *in vitro*" The Journal of Biological Chemistry 259:9805-9812.

Owen, M. (1963) "Cell population kinetics of an osteogenic tissue. 1" Journal of Cell Biology 19:19-31.

Owen, M. (1980) "The origin of bone cells in the postnatal organism" Arthritis and Rheumatism 23:1073-1080.

Owen, M. (1985) "Lineage of osteogenic cells and their relationship to the stromal system" in "Bone and Mineral Research/3" (Peck, W.A. ed.) pp 1-25. Elsevier Science Publishers.

Owen, M. and Ashton, B. (1986) "Osteogenic differentiation of skeletal cell populations" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y. ed.) pp 279–284. Elsevier Science Publishers.

Owen, M., Cavé, J. and Joyner, C.J. (1987) "Clonal analysis *in vitro* of osteogenic differentiation of marrow CFU-F" Journal of Cell Science 87:731-738.

Palumbo, C. (1986) "A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos" Cell and Tissue Research 246:125-131.

Parfitt, A.M. (1988) "Bone remodeling: Relationship to the amount and structure of bone, and the pathogenesis and prevention of fractures" in "Osteoporosis: Etiology, Diagnosis, and Management" (Riggs, B.L. and Melton, L.J., eds.) pp 45-93. Raven Press, New York.

Parry, C.R. (1985) "Osteoblasts are the first bone cells in matrix-induced endochondral bone formation" South African Journal of Science 81:633-635.

Partridge, N.C., Frampton, R.J., Eisman, J.A., Michelangeli, V.P., Elms, E., Bradley, T.R. and Martin, T.J. (1980) "Receptors for 1,25$(OH)_2$-vitamin D_3 enriched in cloned osteoblast-like rat osteogenic sarcoma cells" Federation of European Biochemical Societies Letters 115:139-142.

Patt, H.M., Maloney, M.A. and Flannery, M.L. (1982) "Hematopoietic microenvironment transfer by stromal fibroblasts derived from bone marrow varying in cellularity" Experimental Haematology 10:738-742.

Peck, W.A., Birge, S.J. and Fedak, S.A. (1964) "Bone cells: Biochemical and biological studies after enzymatic isolation" Science 146:1476-1477.

Peck, W.A., Burks, J.K., Wilkins, J., Rodan, S.B. and Rodan, G.A. (1977) "Evidence for preferential effects of parathyroid hormone, calcitonin and adenosine on bone and periosteum" Endocrinology 100:1357-1364.

Peck, W.A. and Woods, W.L. (1988) "The cells of bone" in "Osteoporosis: Etiology, Diagnosis, and Management" (Riggs, B.L. and Melton, L.J., eds) pp 1-43. Raven Press, New York.

Piez, K.A. (1987) "Collagen types: A review" in "Development and Diseases of Cartilage and Bone Matrix" (Sen, A. and Thornhill, T. eds.) pp 1-19. Alan R. Liss, Inc.

Pinnell, S.R., Murad, S. and Darr, D. (1987) "Induction of collagen synthesis by ascorbic acid" Archives of Dermatology 123:1684-1686.

Price, P. (1983) "Osteocalcin" in "Bone and Mineral Research/1" (Peck, W.A. ed.) pp 157-190. Excerpta Medica.

Price, P. (1988) "New bone markers" Triangle 27:21-26.

Prince, C.W. (1987) "Proteoglycans of mineralised matrices" Methods in Enzymology 145:261-269.

Puzas, J.E. and Brand, J.S. (1979) "Parathyroid hormone stimulation of collagenase secretion by isolated bone cells" Endocrinology 104:559-562.

Raisz, L.G. (1988) "Bone metabolism and its hormonal regulation: An update" Triangle 27:5-10.

Raisz, L.G., Dietrich, J.W. and Canalis, E.M. (1976) "Factors influencing bone formation in organ culture" Israeli Journal of Medical Science 12:108-114.

Raisz, L.G. and Niemann, I. (1969) "Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture" Endocrinology 85:446-452.

Raisz, L.G., Simmins, H.A., Sandberg, A.L. and Canalis, E. (1980) "Direct stimulation of bone resorption by epidermal growth factor" Endocrinology 107:270-273.

Rath, N.C. and Reddi, A.H. (1979) "Collagenous bone matrix is a local mitogen" Nature 278:855-857.

Reddi, A.H. (1985) "Regulation of bone differentiation by local and systemic factors" in "Bone and Mineral Research/3" (Peck, W.A., ed.) pp 27-47. Elsevier Science Publishers.

Reynolds, J.J. (1976) "Organ cultures of bone: Studies on the physiology and pathology of resorption" in "Organ Culture in Biomedical Research" (Balls and Monnickendam, eds.) pp 355-366. Cambridge University Press.

Rodan G.A. and Martin, T.J. (1981) "Role of osteoblasts in hormonal control of bone resorption - A hypothesis" Calcified Tissue International 33:349-351.

Rodan, G.A. and Rodan, S.B. (1984) "Expression of the osteoblastic phenotype" in "Bone and Mineral Research/2" (Peck, W.A., ed.) pp 244-285. Elsevier Science Publishers.

Rodan, S.B. and Rodan, G.A. (1974) "The effect of parathyroid hormone and thyrocalcitonin on the accumulation of cyclic adenosine 3':5'-monophosphate in freshly isolated bone cells" Journal of Biological Chemistry 249:3068-3074.

Rodan, S.B., Wesolowski, G., Thomas, K. and Rodan, G.A. (1987) "Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor" Endocrinology 121:1917—1923.

Rosen, D.M., Stempien, S.A., Thompson, A.Y. and Seyedin, S.M. (1988) "Transforming growth factor-beta modulates the expression of osteoblast and chondroblast phenotypes *in vitro*" Journal of Cellular Physiology 134:337-346.

Rubin, H., Hennessey, T.L., Sanui, H., Arnstein, P., Taylor, D.O.N. and Chu, B.M. (1985) "Inheritance of acquired changes in growth capacity of spontaneously transformed Balb/3T3 cells propagated in mice and culture" Cancer Research 45:2590-2599.

Sakamoto, S. and Sakamoto, M. (1986) "Bone collagenase, osteoblasts and cell-mediated bone resorption" in "Bone and Mineral Research/4" (Peck, W.A., ed.) pp 49-102. Elsevier Science Publishers.

Sandberg, M., Autio-Harmainen, H. and Vuorio, E. (1988) "Localization of the expression of types I, III, and IV collagen, TGF-β_1 and c-fos genes in developing human calvarial bones" Developmental Biology 130:324-334.

Schmid, C., Steiner, T and Froesch, E.R. (1984) "Insulin-like growth factor I supports differentiation of cultured osteoblast-like cells" Federation of European Biochemical Societies Letters 173:48-52.

Schneider, G.B. and Relfson, M. (1988) "A bone marrow fraction enriched for granulocyte-macrophage progenitors gives rise to osteoclasts *in vitro*" Bone 9:303-308.

Schneider, G.B., Relfson, M. and Nicolas, J. (1986) "Pluripotent hemopoietic stem cells give rise to osteoclasts" The American Journal of Anatomy 177:505-511.

Schoeters, G.E.R., de Saint-Georges, L., van der Heuvel, R. and Vanderborght, O. (1988) "Minerlization of adult mouse bone marrow *in vitro*" Cell and Tissue Kinetics 21:363-374.

Shiina-Ishimi, Y., Abe, E., Tanaka, H. and Suda, T. (1986) "Synthesis of colony-stimulating factor (CSF) and differentiation-inducing factor (D-factor) by osteoblastic cells, clone MC3T3-E1" Biochemical and Biophysical Research Communications 134:400-406.

Silve, C.M., Hradek, G.T., Jones, A.L. and Arnaud, C.D. (1982) "Parathyroid hormone receptor in intact embryonic chicken bone: Characterization and cellular localization" The Journal of Cell Biology 94:379-386.

Simmons, D.J., Kent, G.N., Jilka, R.L., Scott, D.M., Fallon, M. and Cohn, D.V. (1982) "Formation of bone by isolated, cultured osteoblasts in Millipore diffusion chambers" Calcified Tissue International 34:291-294.

Sminia, T. and Dijkstra, C.D. (1986) "The origin of osteoclasts: An immunohistochemical study on macrophages and osteoclasts in embryonic rat bone" Calcified Tissue International 39:263-266.

Smith, D.M., Johnston, C.C. and Severson, A.R. (1973) "Studies of the metabolism of separated bone cells. 1. Techniques of separation and identification" Calcified Tissue Research 11:56-69.

Sodek, J. and Berkman, F.A. (1987) "Bone cell cultures" Methods in Enzymology 145:303-324.

Somerman, M., Hewitt, A.T., Varner, H.H., Schiffmann, E., Termine, J. and Reddi, A.H. (1983) "Identification of a bone matrix-derived chemotactic factor" Calcified Tissue International 35:481-485.

Sparks, R.L. and Scott, R.E. (1986) "Transforming growth factor type ß is a specific inhibitor of 3T3 T mesenchymal stem cell differentiation" Experimental Cell Research 165:345-352.

Sparks, R.L., Seibel-Ross, E.I., Wier, M.L. and Scott, R.E. (1986) "Differentiation, dedifferentiation, and trans-differentiation of Balb/c 3T3 T mesenchymal stem cells: Potential significance in metaplasia and neoplasia" Cancer Research 46:5312-5319.

Stenner, D.D., Tracy, R.P., Riggs, B.L. and Mann, K.G. (1986) "Human platelets contain and secrete osteonectin, a maker protein of mineralised bone" Proceedings of the National Academy of Sciences 83:6892-6896.

Sterrett, J.D. (1986) "The osteoclast and periodontitis" Journal of Clinical Periodontology 13:258-269.

Stinson, R.A. and Chan, J.R.A. (1987) "Alkaline phosphatase and its function as a protein phosphatase" in "Advances in Protein Phosphatases. Volume 4" (Merlevede, W. and di Salvo, J., eds.) pp 127-151. Levven University Press.

Stracke, H., Schulz, A., Moeller, D., Rossol, S. and Schatz, H. (1984) "Effect of growth hormone on osteoblasts and demonstration of somatomedin-C/IGF I in bone organ culture" Acta Endocrinologica 107:16-24.

Sudo, H., Kodama, H.-A., Amagai, Y., Itakura, Y. and Yamamoto, S. (1986) "Mineralized tissue formation by MC3T3-E1 osteogenic cells embedded in three-dimensional gel matrix" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y., ed.) pp 291-296. Elsevier Science Publishers.

Sudo, H., Kodama, H.-A., Amagai, Y., Yamamoto, S. and Kasai, S. (1983) "*In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria" The Journal of Cell Biology 96:191-198.

Sugimoto, T., Nakada, M., Fukase, M., Imai, Y., Kinoshita, Y. and Fujita, T. (1986) "Effects of ascorbic acid on alkaline phosphatase activity and hormone responsiveness in the osteoblastic osteosarcoma cell line UMR-106" Calcified Tissue International 39:171-174.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Mosely, J.M., Martin, T.J. and Suda, T. (1988) "Osteoblastic cells are involved in osteoclast formation" Endocrinology 123:2600-2602.

Takahashi, T., Kurihara, N., Takahashi, K. and Kumegawa, M. (1986) "An ultrastructural study of phagocytosis in bone by osteoblastic cells from fetal mouse calvaria *in vitro*" Archives of Oral Biology 31:703-706.

Tenenbaum, H.C. (1981) "Role of organic phosphate in mineralization *in vitro*" Journal of Dental Research 60(C):1586-1589.

Tenenbaum, H.C. and Heersche, J.N.M. (1982) "Differentiation of osteoblasts and formation of mineralized bone *in vitro*" Calcified Tissue International 34:76-79.

Tenenbaum, H.C. and Heersche, J.N.M. (1985) "Dexamethasone stimulates osteogenesis in chick periosteum *in vitro*" Endocrinology 117:2211-2217.

Tenenbaum, H.C. and Heersche, J.N.M. (1986) "Differentiation of osteoid-producing cells *in vitro*: Possible evidence for the requirement of a microenvironment" Calcified Tissue International 38:262:267.

Tenenbaum, H.C., Heersche, J.N.M. and Palangio, K.G. (1986) "Control of minerlization of bone *in vitro*" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y., ed.) pp 315-319. Elsevier Science Publishers.

Termine, J.D. (1983) "Osteonectin and other newly described proteins of developing bone" in "Bone and Mineral Research/1" (Peck, W.A. ed.) pp 144-156. Excerpta Medica.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L. and Martin, G.R. (1981) "Osteonectin, a bone specific protein linking mineral to collagen" Cell 26:99-105.

Thesingh, C.W. (1986) "Formation sites and distribution of osteoclast progenitor cells during the ontogeny of the mouse" Developmental Biology 117:127-134.

Tibone, K.W. and Bernard, G.W. (1982) "A new *in vitro* model of intramembranous osteogenesis from adult bone marrow stem cells" in "Factors and Mechanisms Influencing Bone Growth" pp 107-123. Alan R. Liss, Inc.

Tracy, R.P., Shull, S., Riggs, B.L. and Mann, K.G. (1988) "The osteonectin family of proteins" International Journal of Biochemistry 20:653-660.

Triffitt, J.T. (1987) "The special proteins of bone tissue" Clinical Science 72:399-408.

Urist, M.R. (1965) "Bone: Formation by autoinduction" Science 150:893-899.

Urist, M.R., Mikulski, A. and Lietze, A. (1979) "Solubilized and insolubilized bone morphogenetic protein" Proceedings of the National Academy of Sciences 76:1828-1832.

Vaughan, J. (1981) "Osteogenesis and Haematopoiesis" The Lancet, July 18:133-135.

Vaughan, J. (1984) "The influence of bone cells on the formation, and mineralization, of bone matrix" Philosophical Transactions of the Royal Society of London B304:453-454.

Walters, M.R., Rosen, D.M., Norman, A.W. and Luben, R.A. (1982) "1,25-dihydroxyvitamin D receptors in an established bone cell line" The Journal of Biological Chemistry 257:7481–7484.

Weir, E.C., Insogna, K.L. and Horowitz, M.C. (1989) "Osteoblast-like cells secrete granulocyte-macrophage colony-stimulating factor in response to parathyroid hormone and lipopolysaccharide" Endocrinology 124:899-904.

Whitson, S.W., Harrison, W., Dunlap, M.K., Bowers, D.E., Fisher, L.W., Gehron Robey, P. and Termine, J.D. (1984) "Fetal bovine bone cells synthesise bone-specific matrix proteins" The Journal of Cell Biology 99:607-614.

Wong, G.L. and Cohn, D.V. (1974) "Separation of parathyroid hormone and calcitonin-sensitive cells from non-responsive cells" Nature 252:713-715.

Wong, G.L. and Cohn, D.V. (1975) "Target cells for parathormone and calcitonin are different: Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces" Proceedings of the National Academy of Science 72:3167-3171.

Wong, G.L., Luben, R.A. and Cohn, D.V. (1977) "1,25-dihydroxycholecalciferol and parathormone: Effects on isolated osteoclast-like and osteoblast-like cells" Science 197:663-665.

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. and Wang, E.A. (1988) "Novel regulators of bone formation: Molecular clones and activities" Science 242:1528-1534.

Wrana, J.L., Maeno, M., Hawrylyshyn, B., Yao, K.-L., Domenicucci, C. and Sodek, J. (1988) "Differential effects of transforming growth factor-ß on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations" The Journal of Cell Biology 106:915-924.

Wuthier, R.E. (1986) "Mechanisms of matrix vesicle-mediated mineralization" in "Cell Mediated Calcification and Matrix Vesicles" (Ali, S.Y., ed.) pp 47-55. Elsevier Science Publishers.

Yagiela, J.A. and Woodbury, D.M. (1977) "Enzymatic isolation of osteoblasts from fetal rat calvaria" Anatomical Record 188:287-306.

Yokota, K., Kusaka, M., Ohshima, T., Yamamoto, S., Kurihara, N., Yoshino, T. and Kumegawa, M. (1986) "Stimulation of prostaglandin E_2 synthesis in cloned osteoblastic cells of mouse (MC3T3-E1) by epidermal growth factor" The Journal of Biological Chemistry 261:15410-15415.

Zaidi, M., Moonga, B., Moss, D.W. and MacIntyre, I. (1989) "Inhibition of osteoclastic acid phosphatase abolishes bone resorption" Biochemical and Biophysical Research Communications 159:68-71.

Zambonin Zallone, A. and Teti, A. (1984) "A simple method for isolating osteoblasts from mouse calvaria" Bollettino della Societa Italiana di Biologia Sperimentale 60:1549-1555.

Figure 9. (Mag. x 400)

Transmission electron micrograph of the surface of a 14 day old primary culture of neonatal mouse calvarial osteoblasts showing an osteogenic nodule (N). A speckled image of the X-ray emmission of the Kα of calcium was overlaid, showing the localisation of calcium to the nodule.


N

Figure 10.

A plot of the X-ray emmission analysis of a mineralised osteogenic nodule. There are prominent peaks for phosphorous (P) and calcium (Kα), with small peaks for sodium (NA), sulphur (S) and calcium (Kß).

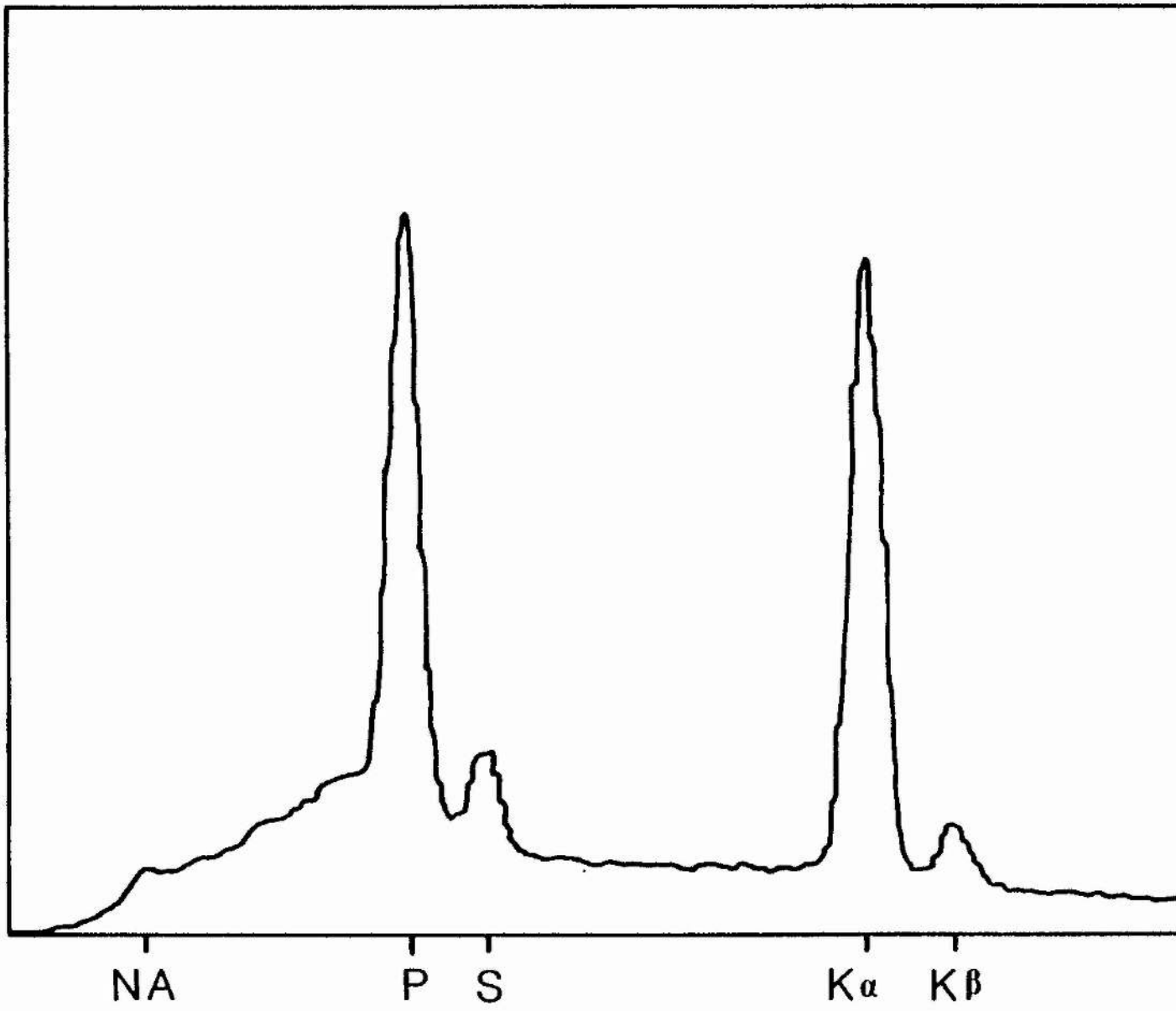

NA
P
S
Kα
Kβ

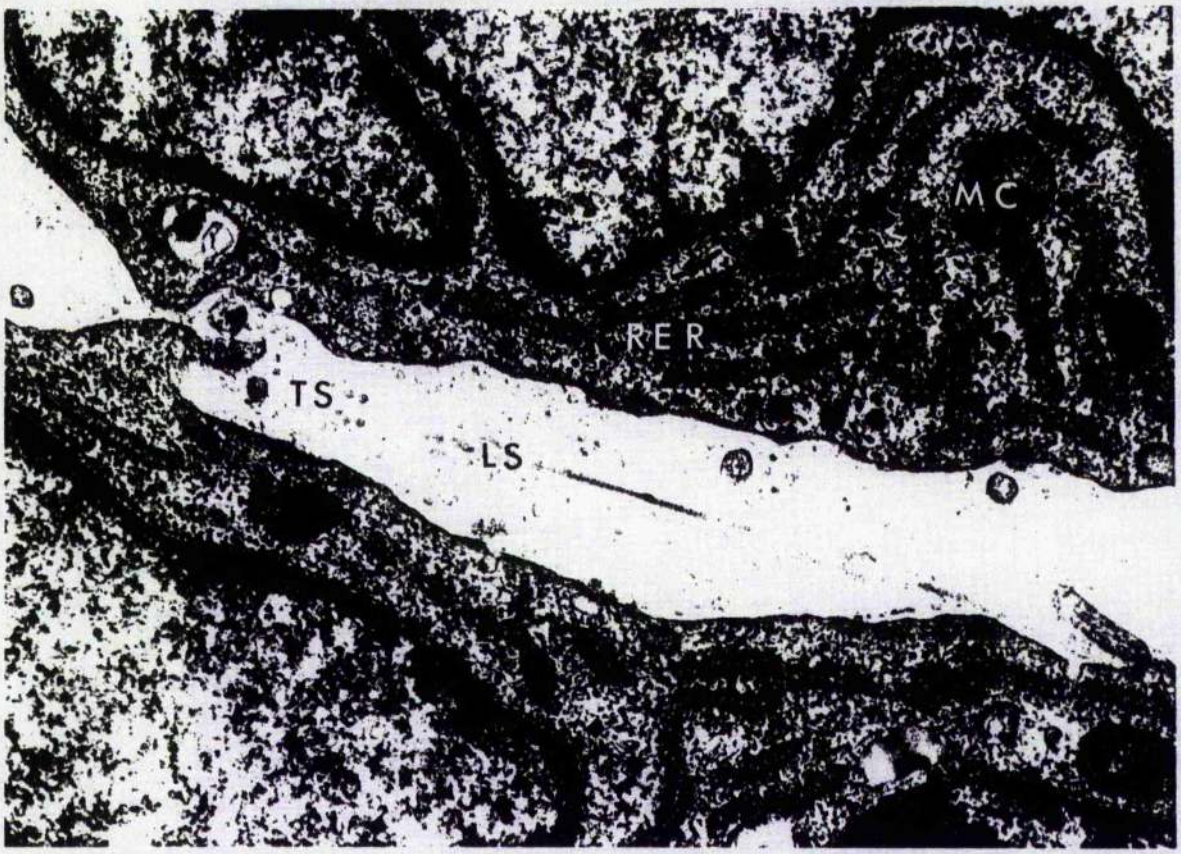
Figure 11. (Mag. x 30,700)

Transmission electron micrograph from a 7 day old culture.

Figure 12. (Mag. x 28,170)

Transmission electron micrograph from a 10 day old culture.

The cultures have started to form multilayers of cells. The cytoplasmic organelles are characteristic of cells involved involved in active protein synthesis; mitochondria (MC) and rough endoplasmic reticulum (RER). The extracellular spaces contains collagen fibrils in transverse (TS) and longitudinal section (LS).


MC
RER
TS
LS

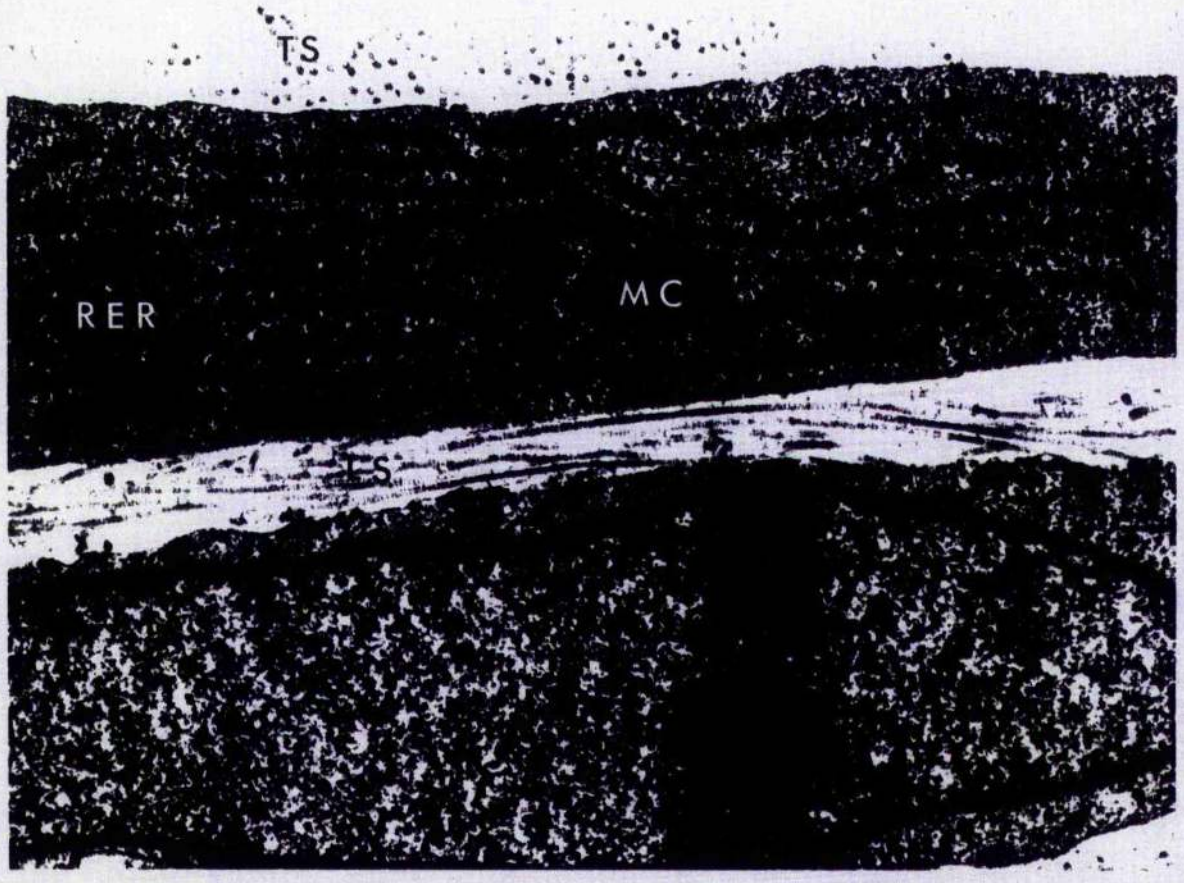

TS
RER
MC
LS

Figure 13. (Mag. x 42,650)

Transmission electron micrograph from a 10 day old culture. The extracellular space contains abundant collagen fibrils in transverse (TS) and longitudinal section (LS). The collagen fibrils appear to be randomly arranged and are interspersed with matrix vesicles (MV).

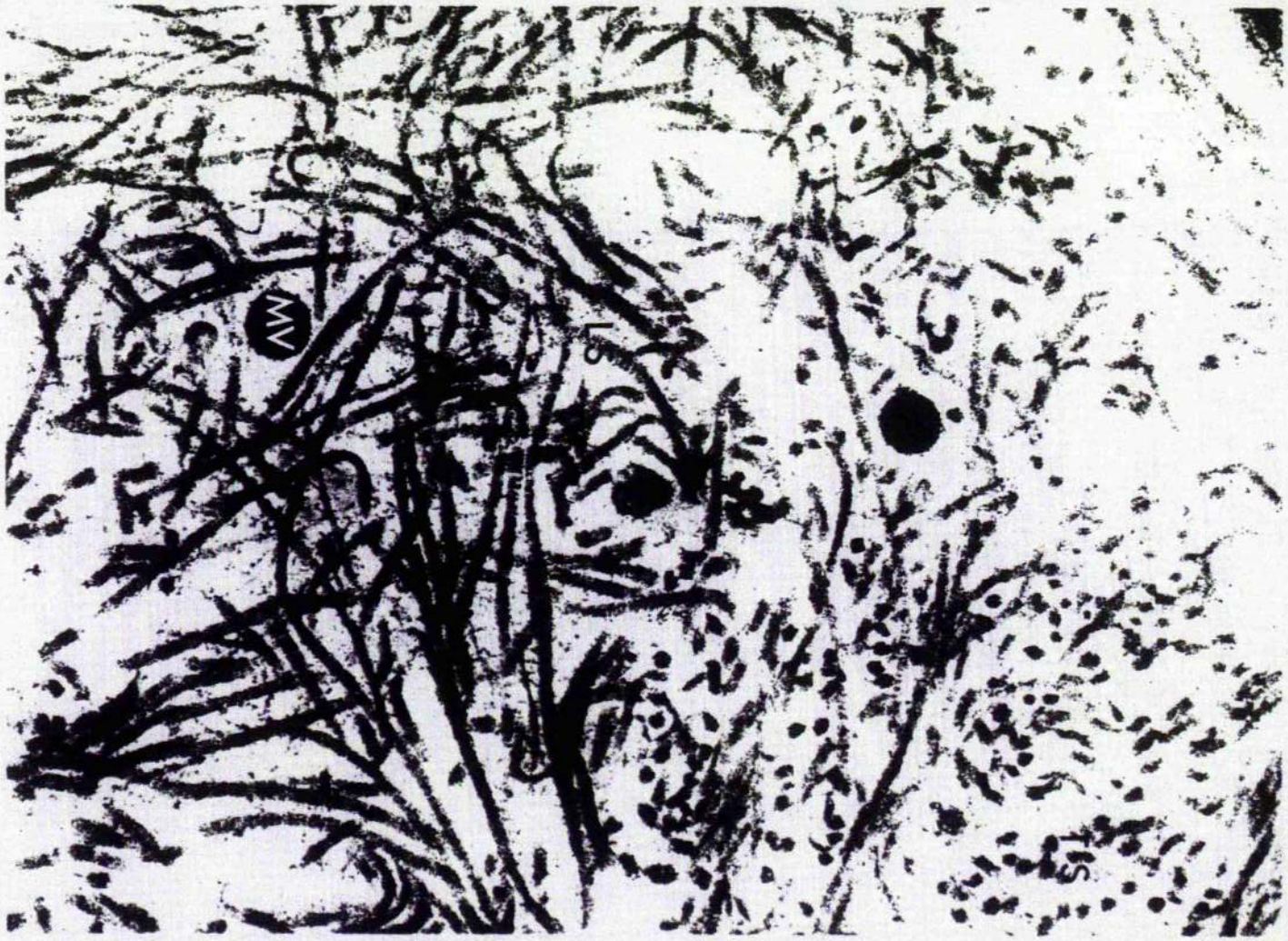


Figure 14. (Mag. x 56,350)

Transmission electron micrograph from a 14 day old culture. Mineral (M) has been deposited on a framework provided by the collagen fibrils (C). Spicules (S) of mineral are extending along the fibrils and lie parallel to the main axis of the fibril. Transverse banding (B) with a periodicity of approximately 62nm is apparent on the fibrils.

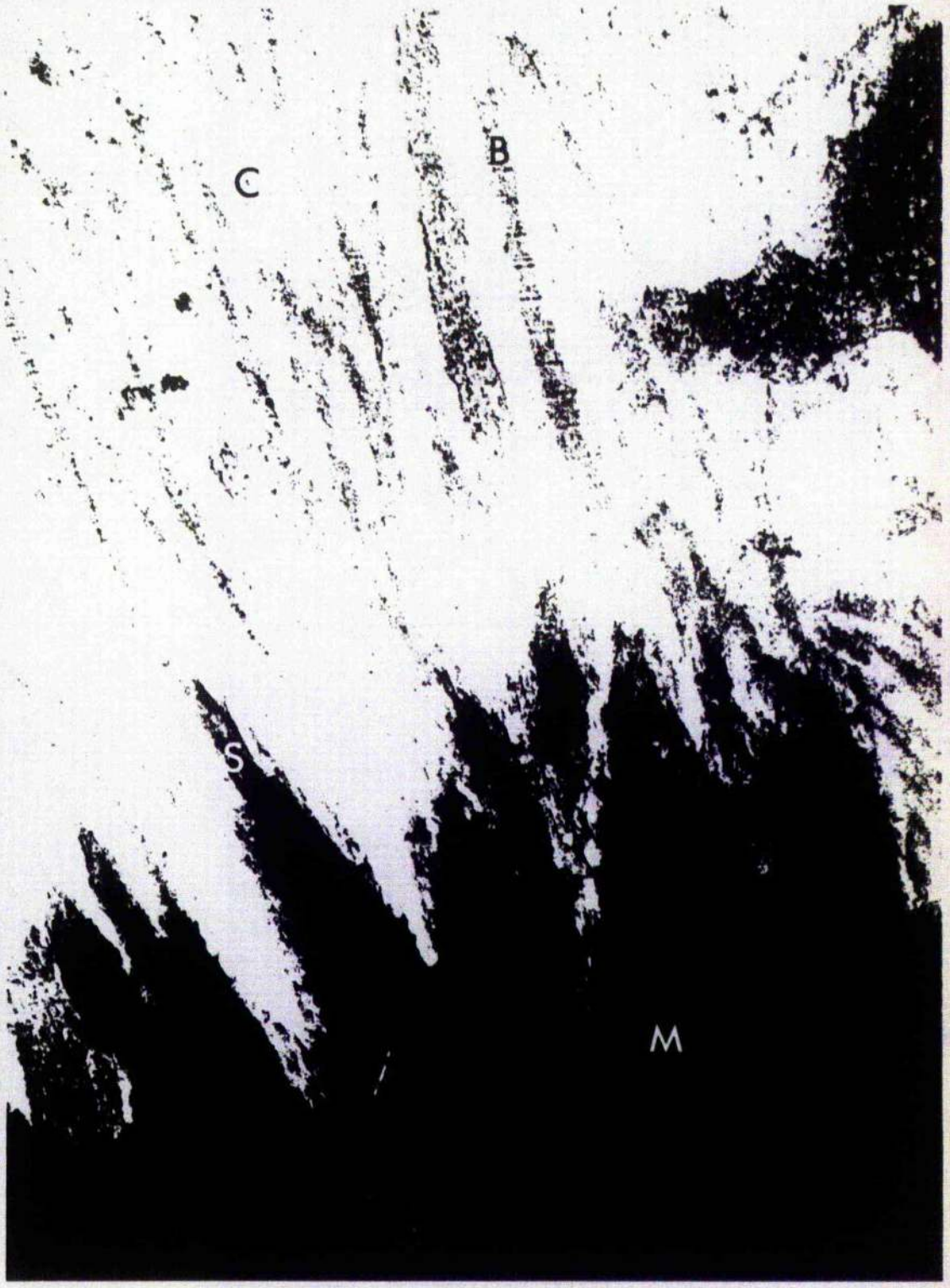

C
B
S
M

Figure 15. (Mag. x 67,050)

Transmission electron micrograph from a 14 day old culture. The extracellular space has been filled with mineral (M). The osteoblasts is still capable of protein synthesis, as shown by the presence of mitochondria (MC) and rough endoplasmic reticulum (RER). Between the cell and the mass of mineral lies an unmineralised seam of osteoid (O).


M
O
M
RER

Figure 16. (Mag. x 10,820)

Transmission electron micrograph from a 14 day old culture. An osteocyte (OC) has become completely surrounded by mineral (M). An unmineralised seam of osteoid (O) is apparent, separating the cell from the mineral. The cell is extending processes (P) towards the mineral, one of which is lying in a canaliculum.

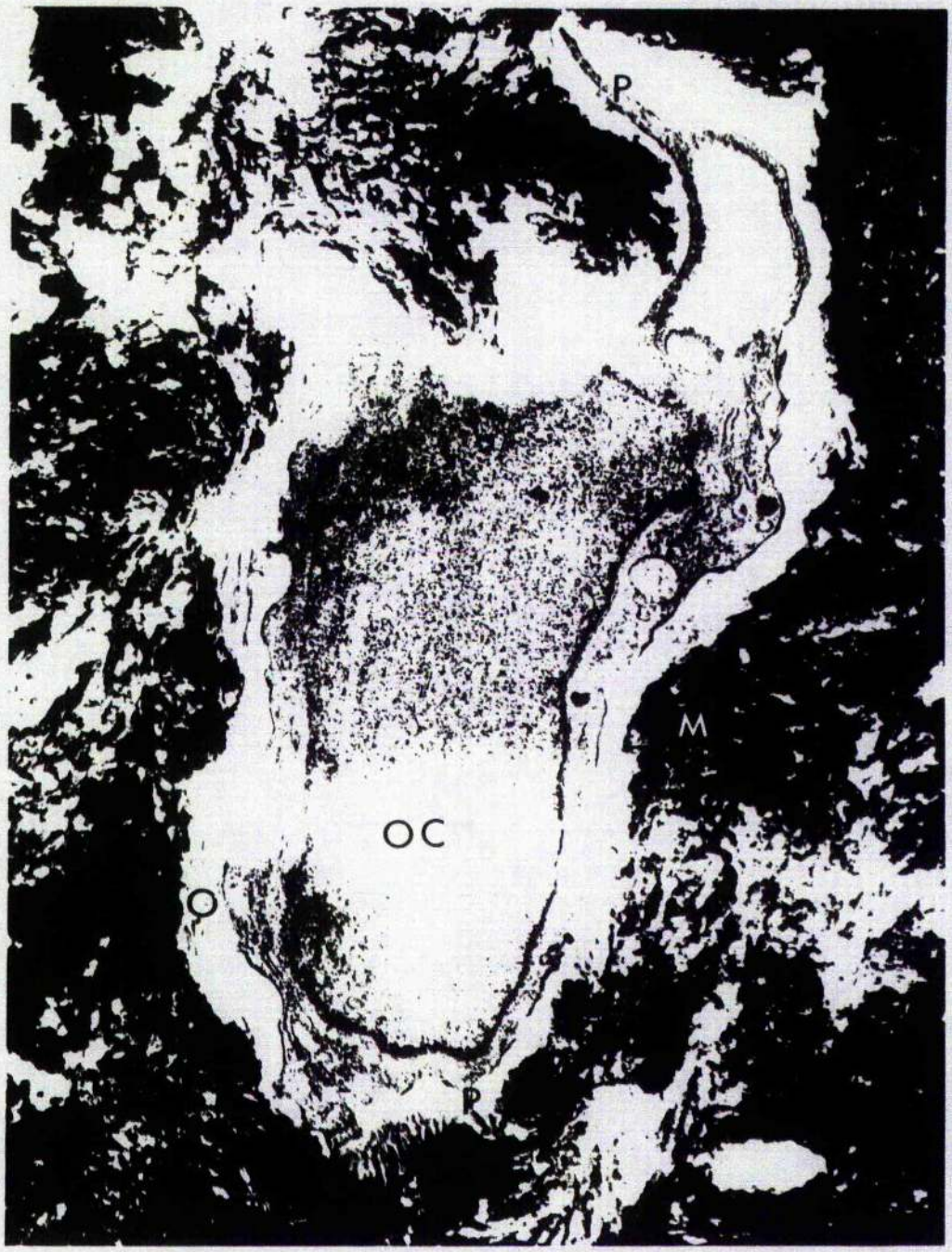

P
M
OC
O
P

Figure 17. (Mag. x 14,250)

Transmission electron micrograph from a 14 day
A rounded cell lying on the surface of a nodule. The
contains mitochondria (MC) and some rough endoplasm
(RER).

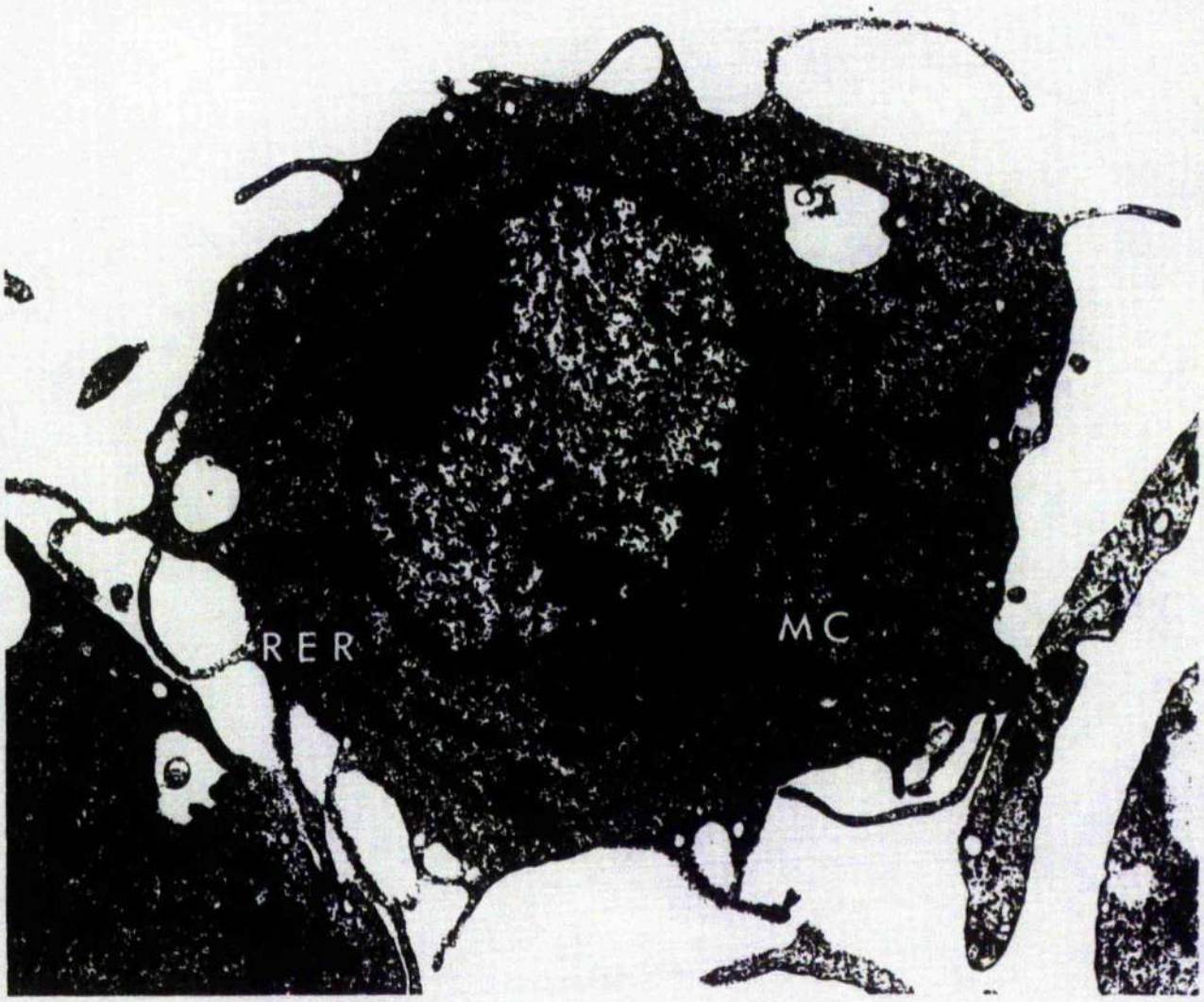

RER
MC

Figure 18. (Mag. x 140)

Phase contrast light micrograph of a culture of 2 to 3 day old primary osteoblasts. The cells have formed a monolayer of polygonal cells.

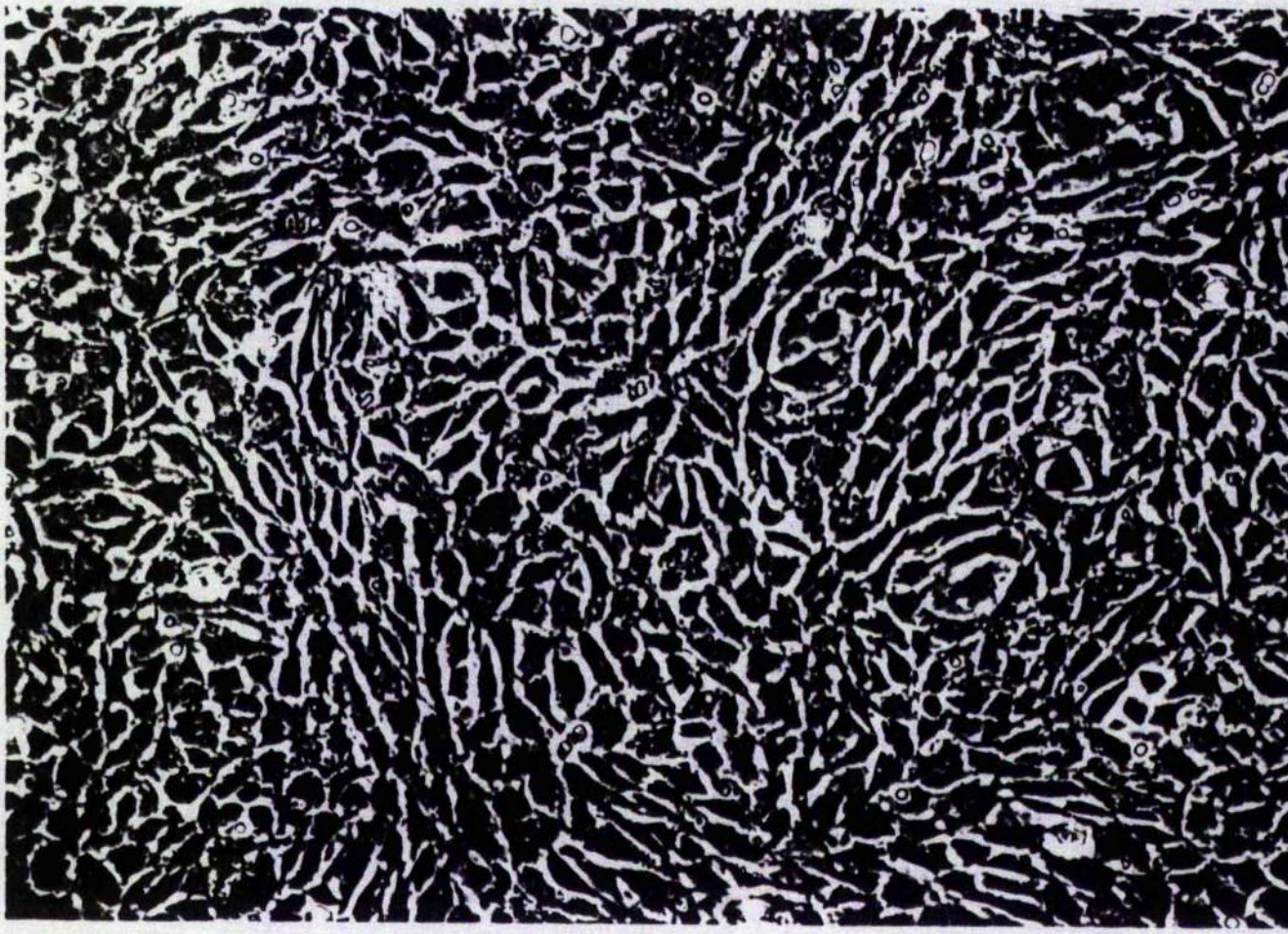


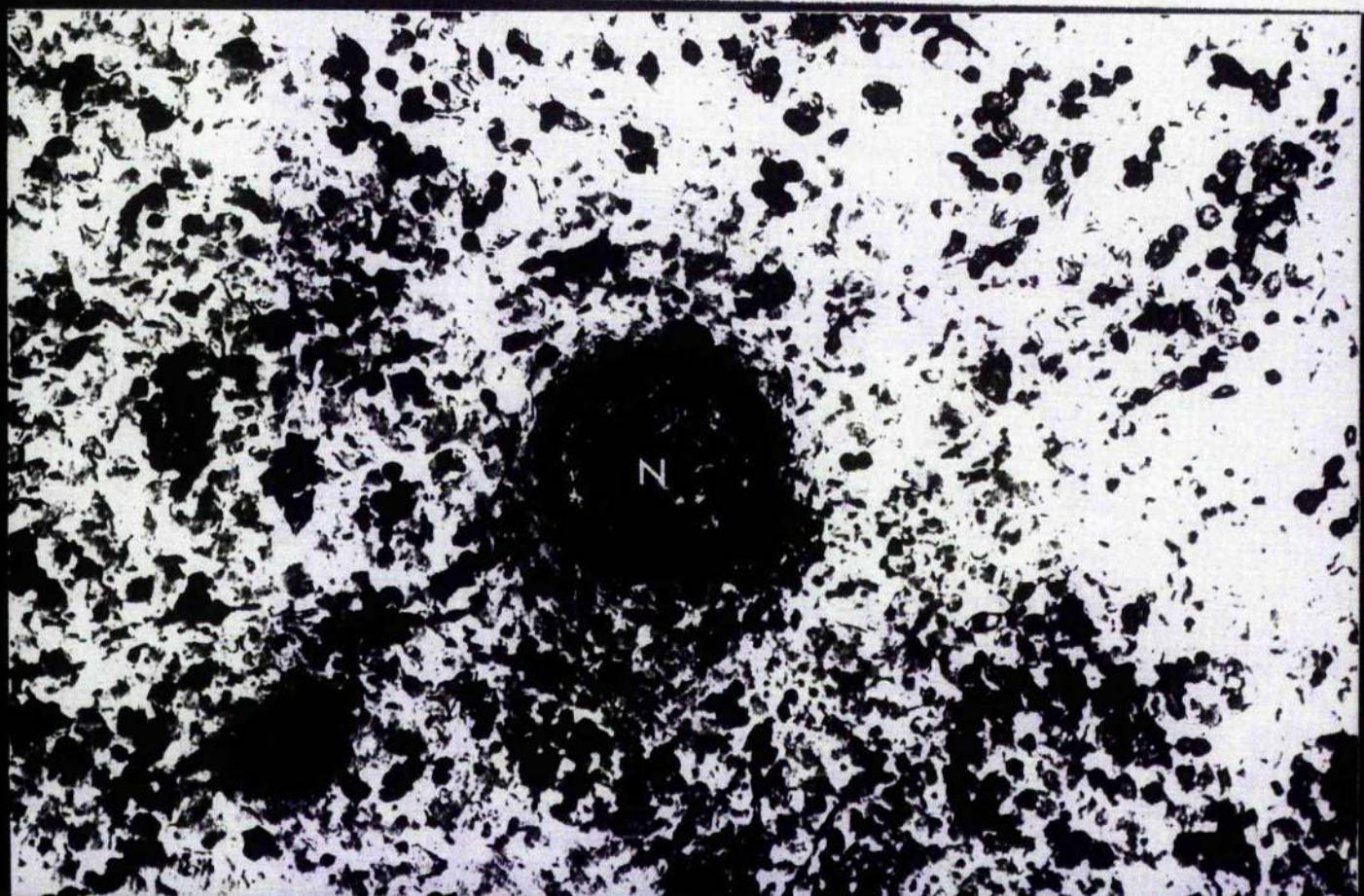
Figure 19a. (Mag. x 160)

Light micrograph of osteoblasts cultured with ascorbic acid and without ß-glycerophosphate. Osteogenic nodules have formed (N). However, these are unmineralised due to the lack of phosphate.

Figure 19b. (Mag. x 160)

Light micrograph of osteoblasts cultured with ascorbic acid and ß-glycerophosphate. An osteogenic nodule (N) has formed and has mineralised. Insoluble calcium is shown by the presence of black deposits of silver.


N
N


N